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INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

Organized: New York City, Dec. 10, 1920. Of those at the meeting of organization, the following remain active members: Adolph Berger, Theodor Blum, G. W. Clapp, A. H. Merritt, B. B. Palmer, L. M. Waugh.

Objects (Const., Art. II): The association has been established to promote broadly the advancement of research in all branches of dental science and in the related phases of the sciences that contribute directly to the development of oral health service, and as a whole; and further, to encourage and facilitate cooperative effort and achievement by, and mutual helpfulness among, investigators in all nations in every division of stomatology to the end that dentistry may render cumulatively more perfect service to humanity.

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Nomination blanks for membership in this Association may be obtained on request from the Secretary-Treasurer.

Endowment fund of the International Association for Dental Research (as of October, 1958): $1,891.01. There have been no expenditures from this fund.

THE JOURNAL OF DENTAL RESEARCH

This JOURNAL is an official publication of the International Association for Dental Research. It is dedicated to the dissemination of knowledge pertaining to the oral cavity, its contents and contiguous parts, and its relation to and effect on the total human organism. The JOURNAL publishes original investigations in these and related fields. Articles are accepted only if they are contributed solely to this JOURNAL.

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INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
ANNOUNCEMENT OF 1960 GENERAL MEETING

The 38th General Meeting of the International Association for Dental Research will convene at the Morrison Hotel, Chicago, Illinois, on Thursday, Friday, Saturday, and Sunday, March 17, 18, 19, and 20, 1960. The Dental Materials Group of the Association will meet on Friday, Saturday, and Sunday, March 18, 19, and 20, 1960. The American Association of Dental Schools will meet at the same place beginning Monday, March 21, 1960.

Notice to Prospective Program Participants.—Abstracts of items for the program must be limited to *250 words,* including title, authors' names, etc. *Three copies* are required. The abstract as received must be suitable for insertion, without editing, in the Preprinted Abstracts which will be sent to members before the meeting. This will not constitute a publication, but is only a convenience for the meeting. The abstracts will be published in the JOURNAL OF DENTAL RESEARCH after the meeting as in the past.

There will be no Read-by-Title section of the program. Instead, the editor is accepting short research notes for early publication in the JOURNAL OF DENTAL RESEARCH.

Abstracts entered in the *Edward H. Hatton Award Competition* should conform to the specifications stated on the opposite page under "Editorial Views and News" and must be clearly marked as entries for the competition.

The deadline for receipt of program items will be *November 30, 1959.* An abstract received after that date will probably not be included in the preprinted abstracts and will appear on the program only if a place can conveniently be found for it.

DAN Y. BURRILL, *Secretary-Treasurer*
Northwestern University Dental School
311 E. Chicago Avenue
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EDITORIAL VIEWS AND NEWS

At the recent and very significant 100th Anniversary Meeting of the American Dental Association and the 47th Annual Session of the Federation Dentaire Internationale, there were several interesting gatherings of related groups. One such gathering was the International Conference on Dental Journalism and Documentation held Sept. 11, 1959, at the New York Academy of Science. It was presented under the joint sponsorship of the Subcommittee on Documentation and the Editorial Board of the F.D.I., the American Association of Dental Editors, the Council on Journalism and the Bureau of Library and Indexing Service of the A.D.A.

This commendable conference was well attended by a vast number of individuals from many different lands, each interested in discussing and improving journalism concerned with the oral area. Presiding officer of the morning session was F. D. Ostrander, President of the A.A.D.E. Past-president Wesley J. Dunn of Canada spoke forcefully on the topic, "The Courage to Be Wrong." Professor A. E. W. Miles of England next discussed "Problems Associated With the Abstracting of Dental Scientific Articles." R. W. McCluggage, Professor of History at Loyola University (Chicago), was a substitute speaker. He described his recent book entitled *The History of the American Dental Association.* Professor Gustav Korkhaus, of Germany, discussed the timely topic of "International Journalism Difficulties Resulting From Different Languages." Completing the morning session was the talk by Richard H. Orr on "Methods for the Improvement of Dental Communication."

LeRoy M. Ennis presided at the luncheon session, and Prof. H. H. Stones of London, Editor of the International Dental Journal, spoke on "Problems of an International Dental Journal."

Presiding at the afternoon session was Morris J. Wilson, Chairman of the A.D.A. Council on Journalism. A symposium on documentation was moderated by C. Gysel of Belgium, Chairman of the F.D.I. Subcommittee on Documentation. One of the most entertaining and constructively critical views was presented by Ida Marian Robinson, who teaches Library Science at the University of Maryland. She spoke about "The Librarian's View of the Dental Journals." Next, Prof. Max Filderman of France discussed in French, "Problems Confronting European Journalism and Documentation." He was followed by Margarita Muruzabal of Argentina, who spoke about "Indexing the Spanish and Portuguese Dental Literature." "Asiatic Dental Journalism and Documentation" was ably discussed by the very aged and erudite Prof. Kazuo Sato of Japan. The final discussion of the afternoon was by Donald A. Washburn, Director of the A.D.A. Bureau of Library and Indexing Service, on "The Index to Dental Literature: Broadening Its Scope and Usefulness."

During the well-attended evening banquet held in the Olympia Room of the Hotel Manhattan, there were presentations of the Distinguished Service Award and the William J. Gies Editorial Award. Speaker of the evening was William H. Laurence, Science Editor of the New York Times, and one of the most significant science writers of the United States.

At the annual business meeting of the A.A.D.E. the following morning, a number of pertinent items were discussed by Sidney Epstein, Secretary-Treasurer, and Floyd D. Ostrander, President. Elected to the Presidency for the coming year was Lon W. Morrey, editor of the J.A.D.A. This concluded an impressive day-and-a-half gathering devoted almost exclusively to journalism in dentistry.

FRANK J. ORLAND
EDITOR

THE EFFECTS OF SALIVA ON THE pH AND LACTATE CONCENTRATION IN DENTAL PLAQUES

I. Caries-Rampant Individuals

HAROLD R. ENGLANDER,* IRVING L. SHKLAIR,** AND LEONARD S. FOSDICK***

The University of Illinois College of Dentistry, The Dental Research Facility USNTC, Great Lakes, Ill., and Northwestern University, School of Dentistry, Chicago, Ill.

INTRODUCTION

THE ENZYME systems in the dentobacterial plaques on human tooth surfaces are capable of producing lactic acid in large quantities from the residual fermentable carbohydrates of dietary origin. When it became possible to measure the pH and lactate content of a given sample of dental plaque, the various factors that may influence plaque pH could be studied. Moore, Carter, Dunn, and Fosdick[1] were the first to measure the pH and lactate concentration in samples of plaque from caries-active individuals before, and at intervals of 5, 10, 15, and 20 minutes after a 50 per cent sucrose mouth rinse. They suggested that the pH and lactate concentration in plaque was a multifunction variable, that is, the acid formed is a function of enzyme systems present, substrate, and temperature. Furthermore, they demonstrated that during the early time intervals following the sucrose mouth rinse, the increase in the per cent of lactate was accompanied by a decrease in pH, and all, or nearly all, of the observed decrease in pH could be accounted for quantitatively by the increase in lactate.

The pH of the dental plaque is a function of: (1) rate of acid formation, (2) rate of acid diffusion out of the plaque, (3) rate of acid neutralization by plaque, tooth substance, and saliva, and (4) the rate of metabolism of lactic acid by microorganisms. Saliva may diffuse into the plaque and substances in the plaque may diffuse out. The rate of neutralization of free lactic acid in plaque would depend upon the buffering capacity of the plaque and the rate of saliva diffusion into the plaque.

In previous studies,[1, 2] it was quite obvious from the pH values obtained by actual measurement that, in all cases, large quantities of the acids formed were immediately neutralized in plaques. This neutralization might have occurred

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*The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Department of the Navy or the Naval Service at large.

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*Department of Therapeutics, University of Illinois, College of Dentistry, Chicago, Ill.

**The Dental Research Facility, Great Lakes, Ill.

***Department of Chemistry, Northwestern University, School of Dentistry, Chicago, Ill.

from the saliva, tooth substance, or from the buffering substances within the plaque itself. The amino acids and amino groups which were present in the plaque might have accounted for some of the neutralization. On the other hand, the washing action of saliva might have been responsible for the rapid clearance of free and neutralized lactic acid from the plaque.

It was the purpose of this study to determine the effect of saliva on the pH and lactate concentration of dental plaque from persons with rampant caries after a 50 per cent sucrose mouth spray, and to define the proportion of lactic acid neutralized in the plaque by saliva and the importance of the washing action of saliva in allowing the diffusion of lactate out of the plaque.

EXPERIMENTAL PROCEDURE

Fifty males, average age 18 years, with rampant caries were selected by means of clinical and radiographic examinations at the U. S. Naval Training Center, Great Lakes, Ill. All had at least 10 or more extensively carious teeth and most had some molar teeth which were decayed down to the gingiva. The majority had decay of the lower anterior teeth.

For each subject, dental plaque pH determinations were made in vitro by means of a microglass electrode in conjunction with a Coleman vacuum tube potentiometer[3] before and at 5, 10, 15, and 20 minutes following a 15-second 50 per cent sucrose oral spray. At each time interval, two plaque pH determinations were made, one each from the buccal surfaces of the upper right and left molars. In most cases, for each individual studied, all samples of plaque were taken from the same surfaces of the same teeth. For purposes of this study, plaque was designated as the organic coating of the tooth, regardless of its physical properties or derivation. This included film, materia alba, and any matter that was consistently found on the tooth surfaces except decay, calculus, and frank food particles.

After the pH was determined, the sample of plaque was placed in 2 ml. of a 1 per cent copper sulfate solution, which stopped enzyme action. This sample was then used for the determination of total lactate by a modification of the method of Barker and Summerson,[4] and for a weight determination.[5]

Each subject returned at the same time on the following day and parotid cups[6] were placed on the right and left Stensen's ducts to prevent parotid saliva from washing over the dental plaque on the upper molars. Strong suction was also used on the floor of the mouth to prevent the submaxillo-sublingual saliva from reaching the upper molars. Plaque pH and lactate determinations were then made in a manner similar to those measured on the previous day. It was thus possible to compare the effect of saliva on plaque pH readings and lactate concentrations in the same individuals following the application of sucrose.

RESULTS

The average of the two plaque pH and lactate values obtained at each time interval from the upper right and left molars was used for each subject.

Figs. 1 and 2 show a plot of the mean pH and lactate values from the 50 caries-rampant subjects before and at 5, 10, 15, and 20 minutes after the 50 per cent sucrose application, with and without saliva restriction. Although the mean pH values before sucrose was applied were approximately the same on both days, the mean actual acidities produced in plaque after the restriction of saliva were greater than when saliva was allowed to flow freely over the plaque. The initial pH of 6.4 fell to 6.0 10 minutes after the application of sucrose, closely approached the initial value at 15 minutes, and was 6.3 at the end of the 20-minute test period (Fig. 1). In contrast to this, in the series of readings after saliva restriction (Fig. 2), the initial pH value was 6.5 but was reduced to 5.6 at the end of the 10-minute interval. This lower pH level was maintained throughout the remainder of the test period.

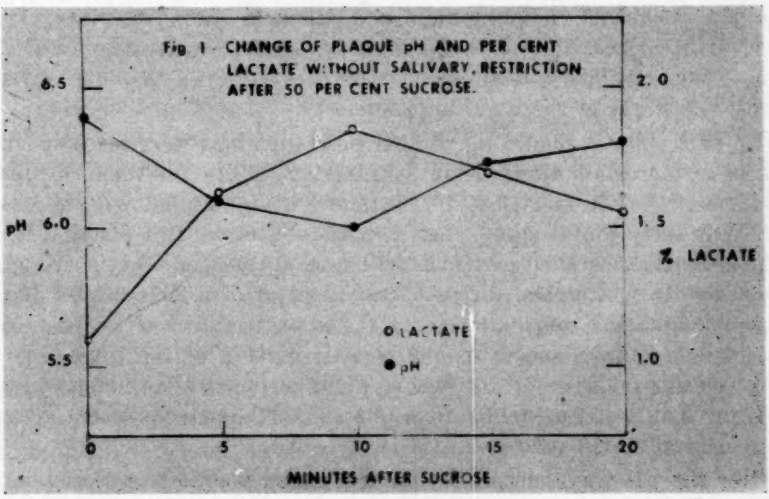

Fig. 1.

Without saliva restriction, the highest lactate value of 1.84 per cent occurred at the same time as the lowest pH value of 6.0, and the lactate concentrations of the plaque began to fall off after 10 minutes. After saliva restriction, the mean per cent lactate rose above the two initial values and remained elevated throughout the 20 minutes. This was accompanied by the reduction of the pH to a persistent level of 5.6.

Since the average pH readings at each time interval present a distorted picture of minimum pH values because individual minimum readings occurred at different time intervals, the 50 subjects were distributed according to the minimum pH values occurring, in either upper molar area, at any time during the 20-minute period (Table I). Whereas, 78 per cent of the subjects with saliva restriction exhibited a minimum pH of 5.5 or below at some time during the test period, only 34 per cent of the subjects without saliva restriction produced values in this range.

TABLE I
DISTRIBUTION OF 50 SUBJECTS ACCORDING TO MINIMUM pH VALUES

	SALIVA		SALIVA RESTRICTION	
	NUMBER OF SUBJECTS	%	NUMBER OF SUBJECTS	%
<5.3	6	12	31	62
5.3-5.5	11	22	8	16
>5.5	33	66	11	22
Totals	50	100	50	100

DISCUSSION

When saliva was prevented from free access to the dental plaque, considerably greater acidities were produced. It is apparent that the saliva was responsible for the prevention of extremely high acidities. Every individual produced greater acidities on the day that saliva was restricted and a greater percentage of this group produced acidities below pH 5.5. Accordingly, in the

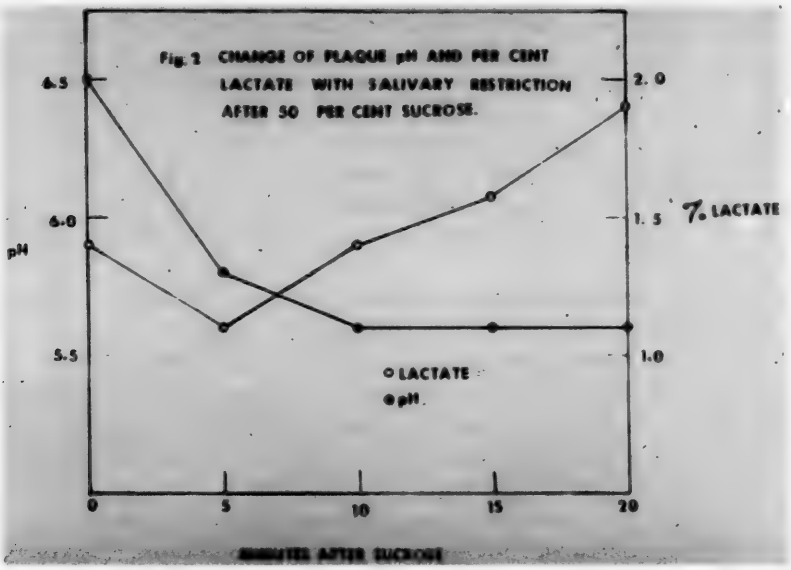

Fig. 2.

restricted group, a greater proportion of the total lactate in the plaque was not neutralized and remained as free lactic acid. Saliva appeared to be responsible for the neutralization of the free acid produced with the consequent rise of the lower plaque pH values to the pre-sucrose level at the end of the 20-minute test period.

The acid produced in the dental plaque was not metabolized appreciably by microorganisms during the 20-minute test period, otherwise the pH would have risen as the acid was being broken down in the restricted group. The pH values remained consistently low in this group. In addition to the buffering action of saliva, there was strong evidence that the plaque substance contributed markedly to this neutralization. In most cases the amount of acid

produced was sufficient to produce pH values of 2 to 3 in a nonbuffered system. Even in the absence of saliva, the pH values never dropped below 4.8. It is obvious, therefore, that the plaque itself had marked buffering capacity, but did not have the capacity to prevent the average pH values from dropping below a dangerous level unless saliva was present. If tooth substance contributed in a major degree to the neutralization of lactic acid, the pH would tend to rise slowly when saliva was restricted. In all probability, if the observations were continued for hours instead of minutes, this would be observed.

Examination of the mean plaque pH and lactate values with saliva showed that the average per cent lactate declined steadily after reaching a peak of 1.84 at 10 minutes and dropped to 1.55 per cent lactate at 20 minutes. This steady fall in lactate was accompanied by a rise in pH from 6.0 at 10 minutes to 6.3 at 20 minutes, indicating that some lactic acid diffused out of the plaque in addition to being neutralized in the plaque. Certainly, if the microorganisms were not responsible for the oxidation of lactic acid, this reduction in mean per cent lactate could be accounted for only by the washing action of saliva. Restriction of saliva was attended by an increase in total lactate concentration and the high value at 20 minutes was in contrast to this value in the saliva group.

SUMMARY

1. Two series of pH and lactate values were determined in the dental plaques from 50 caries-rampant individuals before and at intervals of 5, 10, 15, and 20 minutes following sucrose application, with and without saliva restriction.

2. Every individual produced greater acidities when saliva was restricted and 78 per cent of this group produced acidities of pH 5.5 or below. Only 34 per cent of the saliva group produced acidities in this range.

3. Plaque substance per se appeared to possess considerable buffering capacity and obviated the production of any acidities below pH 4.8. Saliva was necessary, however, to prevent plaque pH values from falling below the level of pH 5.5.

4. When saliva was permitted free access to the dental plaque, the mean per cent lactate concentrations appeared to have an inverse relation to the reduction and subsequent rise of mean pH values after the 10-minute interval. The reduction of lactate after the 10-minute interval indicated that some of the lactic acid diffused out of the plaque in addition to being neutralized in the plaque.

5. Restriction of saliva was attended by a reduction of the mean plaque acidities to the uniform average level of pH 5.6. This was accompanied by an increase in total lactate concentration.

6. Tooth substance did not appear to contribute appreciably to the neutralization of lactic acid during the 20-minute test period.

7. The acid produced in the dental plaque did not seem to be metabolized by microorganisms during the test period.

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ELECTROPHORESIS OF SALIVA

J. H. GELLER, L. HAMES, AND G. H. ROVELSTAD

The Dental Research Laboratory, U. S. Naval Training Center, Bainbridge, Md.

INTRODUCTION

THE successful application of electrophoresis to the analysis of human plasma proteins[1, 2] and to submaxillary mucin[3] has suggested its usefulness for the analysis of saliva. The purpose of this report is to demonstrate and evaluate the electrophoretic pattern of saliva.

REVIEW OF LITERATURE

Electrophoresis has been used (a) to obtain the isoelectric point of separate components of a solution, (b) to compare mobility patterns of different body fluids at the same pH (usually pH 8.6) in order to illustrate variations due to disease or other external influences, or (c) to detect interactions of proteins with each other or nonprotein materials. Electrophoresis of many body fluids has been carried out at pH 8.6 so that the characteristic mobilities of many proteins are known at this pH.[4-8]

The electrophoretic analysis of blood serum at pH 8.6 shows six peaks labeled according to the speed of separation as protein components. The mobilities at pH 8.6 of these components are recorded together with their known isoelectric points and molecular weights.[9]

Similar relationships for a number of proteins have been reported.[10] Many proteins have mobilities up to –6 ($\times 10^{-5}$ in cm.^{2}/volt-sec.), while the pure acids such as hyaluronic or nucleic acid are in the range of –12 to –15 ($\times 10^{-5}$ in cm.^{2}/volt-sec.). The mucoids have an intermediate acidic property and are in the intermediate range of –7 to –11 ($\times 10^{-5}$ in cm.^{2}/volt-sec.). Trypsin is at its isoelectric point at pH 8.6, and therefore does not move in a pH 8.6 buffer. Isoelectric points on the acid side of pH 7.0 can be approximated by the formula:

$$\text{isoelectric pH} = 6.7 - \frac{\text{mobility}}{2.5}$$

where mobility is taken as the absolute figure (i.e., for serum albumin 6.0).

The electrophoretic pattern of a typical mucoprotein has been demonstrated.[11] Several mucoproteins, resolved into their components of albumin, β-globulin, γ-globulin, and free hyaluronic acid (for synovial fluids, vitreous

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humor, and hyalomucoid), or sialoprotein for submaxillary mucoid, have been listed. The difference between submaxillary mucoid and other mucoproteins given is noted in the lack of hyaluronic acid and presence of sialoprotein. The sialoprotein contains 27 per cent acid polysaccharide and its mobility is greater than albumin but slower than pure hyaluronic acid.[12] The variations in serum protein electrophoretic patterns as determined at pH 8.6 for individuals with various medical disorders have been reported.[13]

A paper electrophoretic pattern for whole saliva was developed[14] in 1953 but no attempt was made to identify the three components present. In 1955, a saliva component with lysozyme activity was described, using paper electrophoresis, which migrated toward the cathode at pH 6.9.[15] An electrophoretic pattern of parotid saliva using the moving boundary technic was reported[16] in 1957, demonstrating the presence of seven possible components.

The electrophoretic and ultracentrifugal patterns of parotid saliva were compared.[17] The amylase fraction was identified as the highest peak in the electrophoretic pattern and as a component of $S_{20} = 4.1$ sedimentation rate in the ultracentrifuge. The value corresponds in order of magnitude to a molecular weight of about 50,000.

These electrophoretic studies of saliva as recorded in the literature demonstrate at least three components. The chief characteristics of these components are that amylase is present in the greatest concentration, lysozyme travels toward the cathode at pH 6.7, and submaxillary mucoid travels fastest toward the anode at pH 8.6.

METHOD OF STUDY

Samples of whole saliva, parotid saliva, and extraparotid saliva (i.e., whole saliva free of parotid secretions) were collected in separate refrigerated test tubes by means of paraffin stimulation. The parotid secretions were obtained using separate collecting chambers, the remaining saliva accumulated in the mouth constituting the extraparotid sample.

The samples of whole and extraparotid saliva were centrifuged at 4° C. at 1,200 G for 20 minutes. Thirty milliliters of each secretion were dialyzed in Visking cellulose casings 21 hours or more against 1 L. of distilled water at 4° C. The samples were then pervaporated by suspending in front of a moving fan, tying off periodically as water evaporated in order to maintain turgidity. By this means, the samples were concentrated by approximately a factor of *ten* and then dialyzed against a liter of veronal-veronal sodium buffer, pH 8.6, ionic strength 0.1. After 60 hours the volume of each sample was adjusted to 3 ml. (one tenth original volume) with the buffer and centrifuged at 1,200 G for 20 minutes at 4° C.

Previous to the electrophoresis examination, 0.3 ml. of sample diluted with 2.7 ml. of buffer was examined photometrically, with buffer as the blank, from 320 to 280 mμ to determine the optical density due to protein.

Electrophoresis was then conducted at 2° C. in veronal buffer (pH 8.6, $\mu = 0.1$, at 180 volts and 11.0 Ma. for 60 minutes) utilizing a self-contained Tiselius electrophoresis unit with a modified Philpot-Svenson optical system

and using a conventional 2 ml. Tiselius cell (Perkin-Elmer model 38A). Patterns were recorded at the beginning and at 30- and 60-minute intervals.

When Tiselius performed electrophoresis upon serum proteins, he found that albumin was the fastest component and that globulin consisted of three components which he named α, β and γ globulin, according to their mobilities and solubilities.[18] The globulin was defined as that protein which precipitated in half saturation of ammonium sulfate. The same convention was adopted by others.[19] Also, the same materials have been defined according to their precipitation in sodium sulfate and their electrophoretic mobility at pH 8.6.[20] In the same manner plant and animal proteins have been labeled albumin, α, β, and γ globulin according to their solubilities and electrophoretic mobilities.

The materials studied here have the same solubility characteristics, are nondialyzable and give both the biuret reaction and ultraviolet absorption at 280 mμ. Since their mobilities at pH 8.6 correspond to those found in human, plant, and animal proteins, they have been similarly labeled.

RESULTS

The typical electrophoretic patterns of saliva of 20 different individuals are summarized and illustrated as one pattern each, for parotid (Fig. 1), extraparotid (Fig. 2), and whole saliva (Fig. 3). It will be noted that the parotid secretion typically presents five peaks while the extraparotid and mixed secretion present only three. Occasionally additional peaks are found in the mixed secretion.

According to these findings, six different components have been suggested as being present in saliva. The mobilities of these components have been calculated and compared to reference materials. On the basis of these data, these components have been labeled mucoid, albumin, α-globulin, β-globulin, γ-globulin, and lysozyme as illustrated in Table I. The component labeled

TABLE I

ELECTROPHORETIC MOBILITIES OF COMPONENTS OF SALIVA AND SELECTED REFERENCE MATERIALS USNTC, BAINBRIDGE, MD., 1957

SALIVA COMPONENT	MOBILITY*	REFERENCE MATERIAL	MOBILITY*
Mucoid	−8.6	Hyaluronic acid	−10.7
Albumin	−6.0	Albumin	− 6.0
α Globulin	−4.4	Hemoglobulin	− 4.8
β Globulin	−3.3	α Amylase	− 3.3
γ Globulin	−1.7	—	—
Lysozyme	+2.7	Egg white lysozyme	+ 3.7

*$\times 10^{-5}$ cm.^{2}/volt-sec.

lysozyme did not travel as fast as the lysozyme of egg white under these conditions but this fraction of saliva was recovered from the negative limb of the electrophoresis cell and subsequent assay demonstrated lysozyme activity.

The distribution of the six components separated is illustrated in Table II. It will be noted that the mucoid component is absent in parotid saliva but is present in extraparotid and the mixed saliva. The three components present most frequently in extraparotid and whole saliva are mucoid, albumin, and β-globulin. However, additional components appear occasionally in extraparotid as γ-globulin and α-globulin.

The mixed secretion usually presents three components only. Occasionally α-globulin and γ-globulin, and lysozyme may be identified. However, all six peaks have not been demonstrated here.

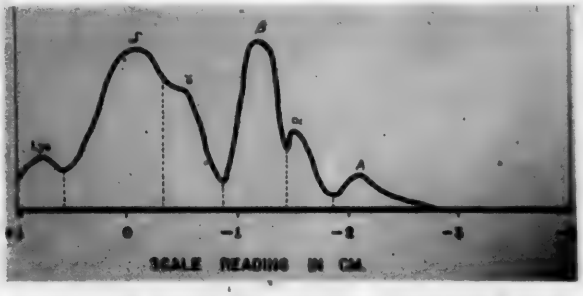

Fig. 1.—Typical electrophoretic pattern of parotid saliva conducted at 2° C. in veronal buffer (pH 8.6, $\mu = 0.1$) at 180 volts and 11.0 Ma. for 60 minutes.

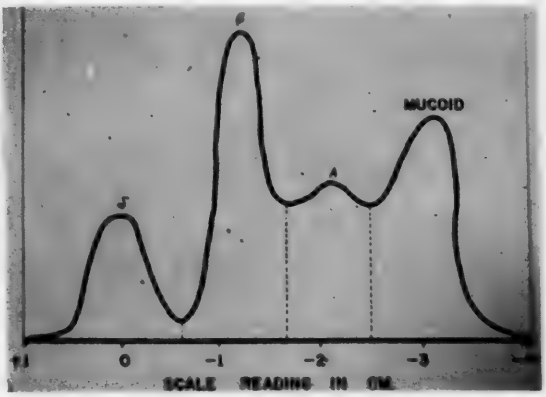

Fig. 2.—Electrophoretic pattern of extraparotid saliva conducted at 2° C. in veronal buffer (pH 8.6, $\mu = 0.1$) at 180 volts and 11.0 Ma. for 60 minutes.

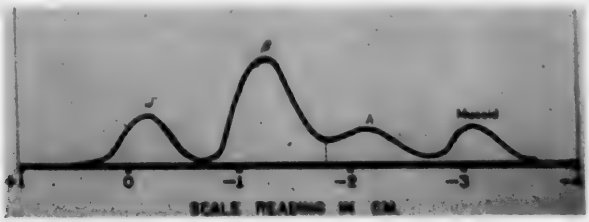

Fig. 3.—Electrophoretic pattern of whole saliva conduced at 2° C. in veronal buffer (pH 8.6, $\mu = 0.1$) at 180 volts and 11.0 Ma. for 60 minutes.

DISCUSSION

A comparison of the typical electrophoretic pattern of saliva with the typical electrophoretic pattern of blood plasma is illustrated in Fig. 4. The relation of the peaks of saliva patterns to those of plasma patterns may be noted. Whereas the albumin component of blood plasma appears in greatest

TABLE II
DISTRIBUTION OF PROTEIN COMPONENTS IN SALIVA
USNTC, BAINBRIDGE, MD., 1957

SALIVA COMPONENT	MOBILITY	SALIVA SAMPLE			APPROXIMATE* ISOELECTRIC POINT (pH)
		WHOLE	EXTRA-PAROTID	PAROTID	
Mucoid (sialo)	−8.6	x	x		2.5
Albumin	−6.0	x	x	x	4.8
Globulin					
α	−4.8	x		x	5.0
β	−3.4	x	x	x	5.5
γ	−1.7		x	x	6.0
Lysozyme	+2.4	x		x	10.8

*Isoelectric pH $= 6.7 - \frac{\text{mobility}}{2.5}$.

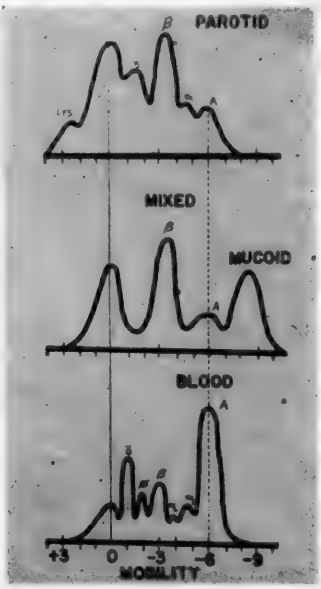

Fig. 4.—Comparison of typical electrophoretic patterns of saliva with typical patterns of blood plasma conducted at 2° C. in veronal buffer (pH 8.6, $\mu = 0.1$) at 180 volts and 11.0 Ma. for 60 minutes.

concentration at pH 8.6, the β-globulin component of saliva appears in greatest concentration. Since this latter component travels the same as α-amylase, at pH 8.6 it contains in all probability the salivary amylase.

The presence of mucoid in extraparotid saliva and not in parotid saliva corresponds to other findings of saliva. The disappearance of α-globulin and γ-globulin and lysozyme from the mixed secretion may be accounted for by this protein being absorbed onto the mucoid and carried with it. It does not seem to be related to the concentration of the total proteins in saliva.

The consistency of patterns from one individual and reproducibility of patterns from a given sample of saliva suggests that this method of evaluating the protein components of saliva can be standardized and related to other factors of dental health.

SUMMARY

Saliva has been evaluated by means of the moving boundary technic of electrophoresis. Six components have been demonstrated as occurring in varying amounts in the different secretions comprising saliva. These components have been labeled according to their mobilities as calculated and as compared to known reference materials.

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DIETARY HABITS AND THE DENTAL CARIES EXPERIENCE IN 200 CHILDREN

ALEGRIA C. ZITA, RALPH E. MCDONALD, AND AARON L. ANDREWS

Indiana University School of Dentistry, Indianapolis, Ind.

MANY studies have been conducted to determine the relationship between dental caries activity and dietary carbohydrates in man. Surveys of the diet of primitive races[1-10] and the diet during wartime[11-13] tend to indicate that the accessibility of refined carbohydrates in the diet is related to the dental caries experience. When readily fermentable carbohydrates are not available, or when they are withdrawn from the diet, the dental caries experience decreases. Numerous studies[14-19] have supported the positive relationship between the readily fermentable carbohydrate in the diet and the dental caries experience. However, some workers have been unable to confirm this observation.[20-23]

The frequency of eating and the amount of carbohydrate consumed between meals also have been considered in previous studies but the results have been contradictory.[24-26] The value of milk as a "protective food" often has been considered, and previous studies have shown that a significant inverse relationship exists between the total milk intake and the dental caries experience.[18, 27] Excessive milk consumption, however, in one study has been associated with a higher dental caries incidence.[26]

The present study was undertaken to obtain additional information about the relationship of dietary habits and the dental caries experience in children.

MATERIAL AND METHOD

Two hundred cases were selected at random from the files of the Pedodontia Department. The survey group consisted of 106 boys and 94 girls, ranging in age from 5 to 13 years. Twenty-five children lived in rural areas, and 175 resided in Indianapolis, or nearby communities. A careful clinical examination was performed by (A. Z. or R. E. M.) with adequate light, mouth mirror, and sharp explorer, after the teeth had been dried with air. A complete roentgenographic survey including bite-wing films was available.

The records of each child included a 7-day diet survey. The parents previously had been requested to keep an accurate record of all foods and the amount consumed at both the mealtime and the between meal period. Only the dietary records that were complete, including the amounts of foods eaten, and those

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that had been prepared with apparent care and interest, were included in this study. Since the role of the diet in dental caries had not been discussed previously with the parents, it was hoped that the diet records were reasonably accurate.

One of the authors (A. Z.) critically reviewed the radiographs of each case to check the accuracy of the recording of carious lesions on the patient's record. In the case of the permanent teeth, the number of affected surfaces expressed as DMFS (decayed, missing, and filled surfaces) was determined. Missing posterior teeth, as a result of extraction, were counted as five surfaces, while a recording of three surfaces was made for each anterior tooth that had been lost as a result of dental caries.

With the aid of the compilations of Bowes and Church,[28] the diet of each child was reduced by calculations to equivalent teaspoons of sugar. One teaspoon of sugar was considered equivalent to 4 grams of refined carbohydrate. In calculating the sugar equivalent in the diet, only the refined foods or those to which sugar had been added were considered. Foods contained in the "Seven Basic Groups," with some exceptions, were not included in the calculation of the sugar equivalent unless free sugar was added during the preparation. One exception was dry cereal. The dried fruits, including raisins, prunes, peaches, apricots, and dates have a very high sugar content; therefore, their sugar equivalents were calculated and included in the total. These are foods commonly eaten by children, particularly between meals, and there is no reason to believe that they are any less cariogenic than refined foods. Although honey is considered as a natural sugar, its sugar equivalent was calculated as were the syrups and other spreads for bread.

The total amount of sugar was calculated for each child and expressed in teaspoons of sugar per week. A tabulation likewise was made for the sugar consumed between meals. Since the frequency of eating may play a role in the dental caries experience, this value was determined for each child. The data from the individual diet survey also were used as an indication of the child's milk consumption and this was recorded as glasses of milk per week.

PRESENTATION OF DATA AND DISCUSSION

The sex of the patient and susceptibility to dental caries was considered since there are conflicting data in the literature on this observation. Klein, Palmer, and Knutson[29] found that girls have a slightly higher DMFS rate than boys, while Potgieter and associates[26] reported no consistent sex difference. In the present study, the mean DMFS was found to be 7.04 for girls and 6.31 for the boys (Table I). This difference was not considered to be statistically significant, at the 65 per cent level of confidence using the critical ratio technic. When the dental status of the rural and city children was compared again, using the critical ratio technic, there was found to be a significant difference at the 4 per cent level of confidence. The mean DMFS for the rural children was 4.92, while that of the city children was 6.9 (Table II).

TABLE I

DIFFERENCE IN DENTAL CARIES EXPERIENCE BETWEEN BOYS AND GIRLS BASED ON DECAYED, MISSING, AND FILLED SURFACES (DMFS)

SEX	MEAN	STANDARD DEVIATION	STANDARD ERROR OF MEAN	NUMBER OF CASES
Boys	6.31	5.84	0.57	106
Girls	7.04	7.65	0.79	94

TABLE II

DIFFERENCE IN DENTAL CARIES EXPERIENCE BETWEEN RURAL AND CITY CHILDREN BASED ON DECAYED, MISSING, AND FILLED SURFACES (DMFS)

	MEAN	STANDARD DEVIATION	STANDARD ERROR OF MEAN	NUMBER OF CASES
Rural	4.92	3.89	0.78	25
City	6.9	7.04	0.53	175

The total weekly sugar intake equivalents were calculated for each of 200 children. The mean total intake was found to be 164 teaspoons, while the mean total between meal sugar was 54.5 teaspoons (Table III).

TABLE III

SUGAR INTAKE EQUIVALENTS OF 200 CHILDREN

	MEAN	STANDARD DEVIATION	RANGE FOR 95%
Total teaspoons of sugar per week	164	17.29	130-200
Total teaspoons of sugar between meals per week	54.51	6.38	42-67

Multiple correlations were made with the DMFS values (Table IV). An attempt was made to determine the relation between total weekly sugar consumption and the dental caries experience. There was found to be a low positive correlation of +0.10, indicating little relationship between these two values. There was found, however, a high positive correlation of +0.77 between the DMFS value and the amount of between meal sugar. A correlation between DMFS and the frequency of between meal eating showed a very low positive correlation of +0.18, which indicates a slight relationship. A correlation was computed to determine the relationship between the DMFS rate and total milk intake, and a very low negative relationship of –0.08 was obtained.

TABLE IV

MULTIPLE CORRELATIONS WITH DMFS VALUES

	CORRELATIONS
Total sugar intake	+0.10
Between meal sugar intake	+0.77
Frequency of between meal eating	+0.18
Milk intake	–0.08

In view of the high positive correlation between DMFS and the between meal sugar, a definitive type of study was done. The upper and lower quartiles

of the between meal sugar data were tested for significance, using the critical ratio technic. This procedure was employed to assist in determining whether the relationship of the two variables was causal or casual. The mean for the lower quartile was 7.79 and the upper quartile was 5.82, as shown in Table V. The resulting test of significance (C. R.) showed the difference not to be statistically significant at the 10 per cent level of confidence. This was interpreted to indicate that, while a high positive correlation exists between these variables, the definitive test on the upper and lower 25 per cent of the cases did not prove to be statistically significant. In view of the variables involved, it appears that there is need for further investigative work in this area.

TABLE V

CORRELATION OF LOWER QUARTILE AND UPPER QUARTILE OF BETWEEN MEAL SUGAR INTAKE AND DMFS

	NUMBER OF CASES	MEAN	STANDARD DEVIATION	STANDARD ERROR OF MEAN
Lower quartile	50	5.82	5.95	0.84
Upper quartile	52	7.79	6.99	0.97

DISCUSSION

It was observed in this study that approximately one third of the weekly total sugar intake, or an average of 55 teaspoon equivalents, was consumed in the form of between meal snacks. The total sugar intake equivalent was high in most children; however, there was little relationship between the total sugar consumed and the dental caries experience. This suggests that dental caries activity was not greatly influenced by a high total sugar intake as long as the sugar was taken at mealtime.

A positive correlation was noted between the DMFS incidence and the amount of sugar consumed between meals. This possibly could be explained by the fact that since one third of the total sugar intake was in the form of between meal snacks, there was a continuous concentration of fermentable carbohydrates in the mouth, and this continuous concentration of substrate favored the production of acids of sufficient potential to produce more carious lesions.

Observations made in previous studies have suggested that with an increased frequency of eating, particularly between meals, there would be an increase in the dental caries experience. In the present study, however, there was found to be very little relationship between the frequency of eating and DMFS. In a review of the dietary records, it was found, however, that the between meal snacks were not always of the cariogenic type. It may be further theorized that inaccuracies in the dietary records, if they do occur, are most likely to be in the recording of between meal snacks, particularly those consumed away from home. In view of this, perhaps relatively little importance should be placed on this observation.

Some investigators have shown that an increased milk intake beyond "reasonable amounts" is associated with an increase in dental caries. This association has been attributed to the fact that the appetite of the child drinking large

quantities of milk will become readily satisfied and there is a tendency to omit the basic portion of the meal and eat only the softer, "more attractive," high carbohydrate foods. This child gets hungry more readily and, as a consequence, there is a greater desire for between meal snacks. The results of the present study, however, did not support this observation. Further studies in this area are needed to clarify the conflicting results.

SUMMARY AND CONCLUSIONS

A study has been undertaken to accumulate additional evidence relative to the effect of dietary habits and the dental caries experience of 200 children.

1. The mean weekly total sugar intake equivalent was 164 teaspoons, while the mean between meal sugar intake equivalent was 55 teaspoons.

2. There was found to be very little relationship correlation between the total sugar consumed and DMFS (+0.10).

3. The total between meal sugar showed a high positive correlation with the DMFS (+0.77).

4. In the present study, there was found to be very little relationship correlation (+0.18) between the frequency of eating and DMFS.

5. There was a slight negative relationship correlation (–0.08) between the total milk intake and DMFS.

6. In equal numbers of children of the same ages, the mean DMFS of the girls (7.07) was higher than the mean DMFS of the boys (6.31). The difference, however, was not considered to be statistically significant using the critical ratio technic.

7. The children residing in the city had a higher DMFS (mean 6.9) than the rural children of comparable ages (mean 4.92). The resulting tests (C. R.) indicated a significant difference in the dental caries experience between the rural and city children at the 4 per cent level of confidence.

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CORRELATION BETWEEN NERVOUS ACCOMMODATION, SYMPTOMATOLOGY, AND HISTOLOGY OF NORMAL AND PATHOLOGIC TOOTH PULP: ITS APPLICATION TO ELECTRODIAGNOSIS

FERNANDO VARGAS F,* AND LUZ VIVALDI M

Instituto de Fisiología y Cátedra de Patología General, Escuela Dental, Universidad de Concepción, Concepción, Chile

AS THE excitability of nerves is altered by environmental changes,[1] pathologic tissues may be diagnosed by measuring a neurophysiologic characteristic whose variation can be detected in the nerves involved. Measurement of nerve thresholds with direct or alternating currents has been, up to now, the procedure most used to diagnose tooth pulp disturbances.[2-7] The great variability of normal tooth thresholds[2] and the lack of parallelism between threshold changes and pulp disturbances[2-3] make greater accuracy unattainable with these methods. The chronaxy of the pulp-sensitive elements also has been studied as a possible method of diagnosis, but no difference has been found between the values of normal and pathologic teeth.[8]

Nervous accommodation, which consists in the progressive elevation of thresholds as the rise-time of stimuli is lengthened, has been measured in the sensitive elements of the tooth and shown to be a linear phenomenon varying significantly in pathologic teeth.[9] The function being linear, it is possible to determine accommodation by measuring only two thresholds with two currents of different time-constants; the ratio between threshold voltages, $\frac{V_2}{V_1}$, is the accommodation index and has been used in the diagnosis of muscular,[10] vestibular,[11] and pulpal disturbances.[12]

The exponentially increasing currents used to measure accommodation are not subject to deformation when passing through tissues,[13] as are rectangular currents,[14] and are unlikely to produce pain.[9-12] The factor of security to pain, i.e., $\frac{\text{pain threshold} \times 100}{\text{sensitive threshold}}$, has been measured for different currents applied to the teeth.[12] The following values have been obtained alternating 17 per cent, direct 117 per cent, and exponentially increasing currents 400 per cent. The purposes of the present investigation were (a) to apply the accommodation index to the diagnosis of different pulp disturbances, (b) to

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*Present Address: Instituto de Fisiología, Universidad de Chile, Casilla 6524, Santiago, Chile.

compare the efficiency of this method with that of clinical diagnosis based on symptoms, and (c) to study the relation existing between the different pathologic states of the pulp, the production of symptoms, and the decrease of nervous accommodation.

A correct comparison between different methods of diagnosis is only possible by checking the diagnosis with the histologic examination of the tissues under study; therefore, our material has been histologically controlled in its entirety.

MATERIALS AND METHODS

The measurements were carried out on 186 teeth from patients of the Immediate Prosthesis Department of the Dental School, the ages ranging between 20 and 60 years. As all the teeth had to be extracted according to prosthetic indication, both normal and pathologic material could be obtained. Such data as were of interest for our study, namely, age of the patient, physical state of the tooth, clinical symptomatology, dental formula, state of the periodontium, and patient's subjective sensation, were entered on a card. Routine x-ray photographs were taken in each case, low-speed films being used.*

The tooth to be examined was carefully isolated by means of cotton, and the surface was dried. The active electrode (cathode) was then fixed to the tooth with Plasticine. The inactive electrode (anode) was tied on the forearm. After this, stimulation commenced with the fastest current, E_1; once the threshold was established, stimulation with E_2 proceeded. The weakest sensation elicited in the patient, and which he indicated by a movement of his hand, was considered as the threshold. An average of 8 measurements of the accommodation index were taken per tooth, the mean value being considered as the corresponding index of the tooth examined.

For the purpose of the clinical diagnosis, the symptoms were ranked schematically on the basis of Euler and Meyer's[15] histopathologic classification in the following way: (1) hyperemia—short pain to cold and heat, and (2) pulpitis—prolonged pain to cold and heat, spontaneous pain. For the present investigation it was of no interest to make subtle distinctions between the clinical diagnostics; the main object was to determine whether or not the clinical method indicated a pulpal disturbance. Pain to percussion was interpreted as a sign of a complication in the pulpal process; if this symptom appeared isolated from others, we diagnosed the case as doubtful, as the sensibility to percussion might result from general disorders.

The stimulating apparatus was based on the same general principle as that designed by Lapicque,[16] with a few modifications, such as a higher internal resistance in order to reduce the variations of tooth resistance. The exponential currents produced, E_1 and E_2, had a rise time of 5 and 16 mSec., respectively. The power source was a charged condenser. The discharge current for stimulation passed through another condenser of convenient capacity to modify its

*A General Electric instrument CDX of the dental type, with Kodak films, was used.

form; adding a second condenser in parallel, which could be connected when desired, a second exponential current was obtained (Fig. 1). Periodical controls of this instrument with a cathode ray oscilloscope has proved the accuracy of its values. The electrodes and other technical details were the same as those described in a previous paper.[9]

The histologic diagnosis of the teeth was carried out in the Department of Pathology of the Dental School. The pulps were fixated in a 20 per cent formalin solution which reached the pulp through the cavities existing in the majority of the teeth. In other teeth, the apex was cut.

Our previous experience and the actual results have assured us that this method gives satisfactory fixation. After an adequate length of time, decalcification in 5 per cent nitric acid, using the technique of Vivaldi,[17] was effected,

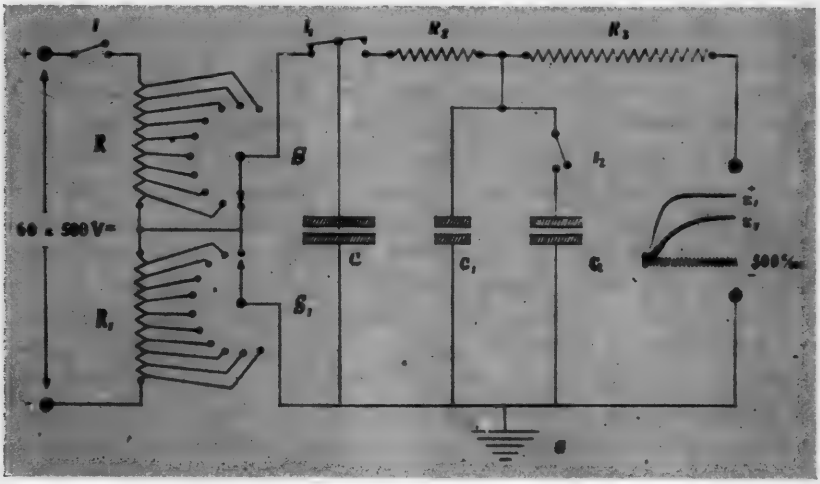

Fig. 1.—Schematic representation of the stimulator. The potentiometers S and S_1 regulate DC voltage. Key I_1 closes the circuit. Condenser C is the power source which discharges when key I_1 is closed. Condenser C_1 produces a delay of the rise-time of discharge current and C_2 is connected through key I_2 when a more delayed current is needed. E_1 and E_2 are the two exponentially increasing currents which the instrument produces (cathode ray oscilloscope tracings).

slight modifications being introduced in accordance with the size of the different teeth. Subsequently, longitudinal sections of the entire tooth were made by means of the congelation microtome. These sections were 10 microns in width, or thinner, when the material permitted. Finally, the sections were systematically stained with hematoxylin-eosin. Only in a few special cases were other stains used. An average of 40 sections per tooth were made.

The teeth were classified in 3 groups according to the histologic picture of the pulps: I—normal pulps, and pulps with slight histologic changes, II—partial diffuse pulpitis, and III—serious pathologic processes. These 3 groups were further divided into subgroups on the basis of microscopic characteristics. In grouping the teeth according to the seriousness of the pulp processes, the main factors taken into account were (a) extension and type of lesion, (b) existence

of circulatory imbalance, and (c) probable prognosis. Teeth with pulpal necrosis were grouped separately. Microphotographs which represent the most typical histologic picture corresponding to each group are shown.

Group I included those teeth with sound pulps (Subgroup 1), teeth exhibiting reticular atrophies (Subgroup 2), and teeth in which the pulps presented very minor tissue reactions to shallow cavities (Subgroup 3).

In Subgroup 2 were included all the processes characterized by reticular atrophy of the pulp and vacuolar atrophy of the odontoblasts. In these teeth the pulp was gradually transformed into a network of conjunctive tissue, and between its meshes were found nerve fibers and blood vessels (Fig. 3). Varying degrees of atrophy were found among the 27 cases in this subgroup, but the nerve response was always similar, except in 1 case of almost total atrophy, in which there was no response. We observed that these pulps maintained their ability to form secondary dentine, even in teeth with a marked atrophy of odontoblasts.

Subgroup 3 included teeth characterized by incipient inflammatory foci. The pulps of these teeth showed limited tissue reactions to shallow cavities. The histologic picture was characterized by the formation of secondary dentine in the zone opposite the position of the caries, under which a scant number of inflammatory cells, particularly lymphocytes, were found. The whole pulp in every case appeared entirely normal except for the small involved zone (Fig. 4).

Group II included teeth exhibiting partial diffuse pulpitis, histologically characterized by the presence of a very thin inflammatory exudation which, however, spread over part of the pulp (Fig. 5).

Group III, serious pathologic processes, included teeth exhibiting pulp disturbances seriously threatening the vitality of this organ. In all of these pulps the circulatory system was altered. Subgroup 1 contained cases of hyperemia in which the pulps showed a marked vasodilation and edema (Fig. 6). Some of these pulps presented light inflammatory processes or cavities, but in all of them the main and more intense disorder was total hyperemia. Subgroup 2, serious degenerative processes, contained pulps affected by hyaline degeneration, fibrosis, calcification, and a very marked dilation of the radicular veins (Figs. 7 and 8). Subgroup 3, containing cases of serious diffuse pulpitis, exhibited advanced pulpal inflammation, with hyperemia, intense exudation and an abundance of inflammatory cells infiltrating a large area of the pulp (Fig. 9).

RESULTS

Accommodation Indices and Symptoms.—Once the accommodation indices of the different subgroups were estimated, a correlation with the histologic classification was observed. The values of the accommodation index decreased as the pathologic states of the pulp became more intense (Fig. 10). In order to establish the limits of the accommodation index corresponding to each group, we added to, or subtracted from, the mean value three times the standard error of the mean. In accordance with this criterion, the index for the first group was above 1.47, that of the second group was between 1.39 and 1.47, and that of the third group was below 1.39. The indices of the subgroups belonging to the first group were significantly higher than those of the third group ($P<0.001$).

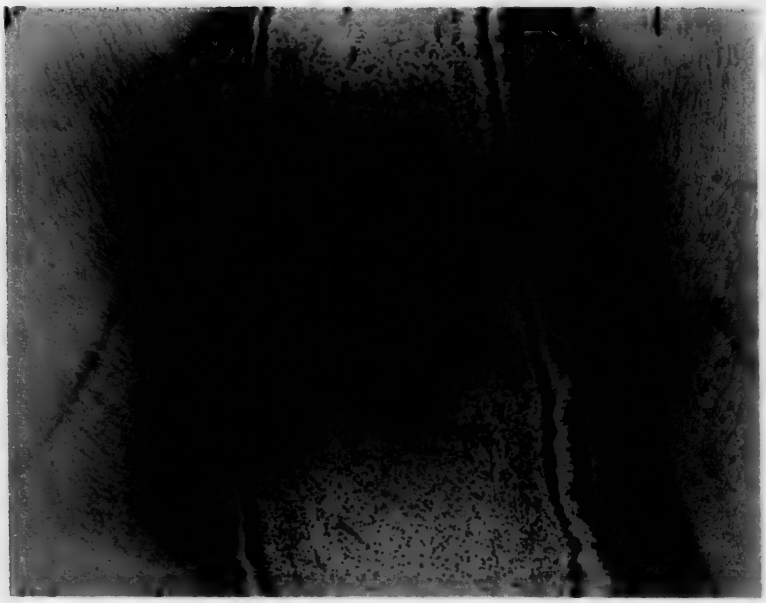

Fig. 2.—Normal pulp (magnification ×49).

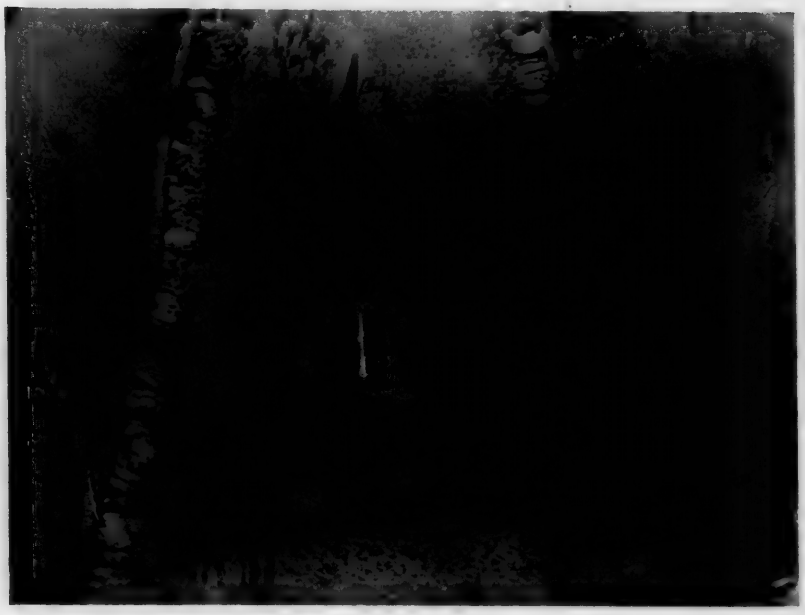

Fig. 3.—Reticular atrophy of the pulp and vacuolar atrophy of the odontoblasts (magnification ×53).

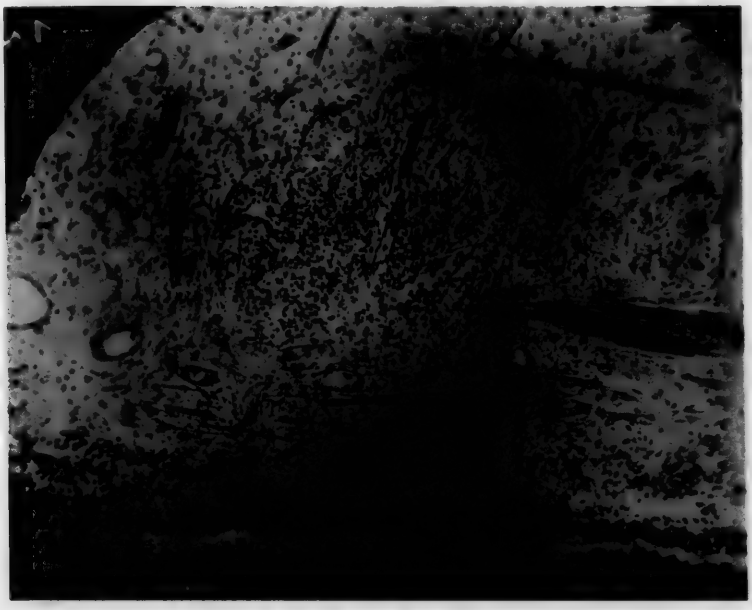

Fig. 4.—Small inflammatory focus in the crown pulp. Secondary dentine may be observed near the focus (magnification ×80).

Fig. 5.—Partial diffuse pulpitis with a slight reticular atrophy. The exudation is formed by plasma cells, lymphocytes, and some polymorphonuclear leukocytes (magnification ×49).

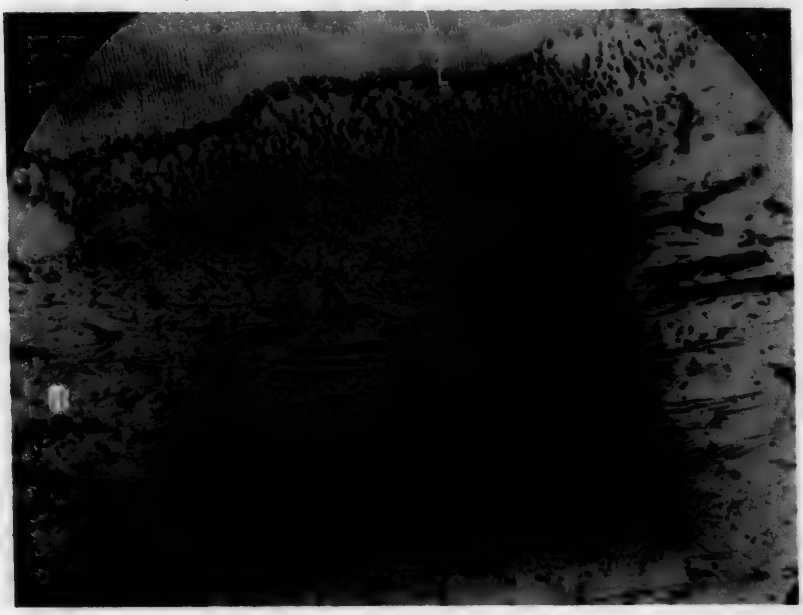

Fig. 6.—Hyperemia and edema, specially marked in the crown pulp (magnification ×135).

Fig. 7.—Radicular pulp showing venous dilation, calcification, and hyalinization (magnification ×87).

The indices of the subgroups classified in the first and third groups, when compared to the index of the second group, showed a statistically significant difference; only cases of hyperemia showed a probable difference (Fig. 11). The percentage of teeth presenting a lowered accommodation ranged from 71 to 75 per cent for the different pathologic states, increasing only in serious diffuse pulpitis to 88 per cent.

Fig. 8.—Crown pulp of the same tooth of Fig. 7 showing marked reticular atrophy, vacuolar atrophy of odontoblasts, calcification, hyalinization, etc. (magnification ×31).

Fig. 9.—Serious diffuse pulpitis (magnification ×50).

Pain frequency amounted to 35 per cent in partial diffuse pulpitis, 29 per cent in cases of hyperemia, 6 per cent in serious degenerative processes and 44 per cent in serious diffuse pulpitis. Since the decrease of accommodation was more frequent than clinical symptoms, it appeared that the method based on accommodation was more efficient than the clinical method in diagnosing pulp disturbances (Table I). Some errors in the clinical diagnosis of teeth of the first group were due to referred pain evoked by another tooth. We speak of a correct diagnosis when it revealed actual pulpal abnormality, without pretending an accurate differentiation in the varied existent pathologic states.

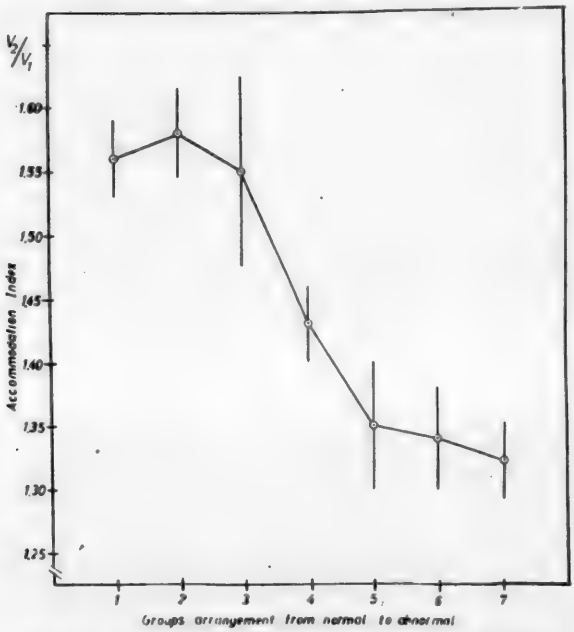

Fig. 10.—Decrease of the accommodation index as the pulp becomes abnormal. *1*, Normals. *2*, Reticular atrophies. *3*, Incipient inflammatory foci. *4*, Partial diffuse pulpitis. *5*, Hyperemias. *6*, Serious degenerative processes. *7*, Serious diffuse pulpitis.

TABLE I
DIAGNOSTIC EFFICIENCY IN CLINICAL AND ELECTRIC METHODS

GROUP	NUMBER OF CASES	CORRECT DIAGNOSIS		EFFICIENCY (PER CENT)	
		CLINICAL	ELECTRIC	CLINICAL	ELECTRIC
Group I					
1	24	23	23	96	96
2	27	25	24	92	89
3	11	11	8	100	73
Group II	17	6	12	35	71
Group III					
1	7	2	5	29	71
2	16	1	12	6	75
3	34	15	30	44	88

The x-ray examination was ineffectual as a direct method of diagnosis but useful as an indirect one in the diagnosis of pulpal alterations.

Of the 35 teeth with pulpal necrosis, 20 were diagnosed by microscopic examination and 15 by clinical means. In some teeth a vague and weak response was found, indicating a stimulation of the periodontium. These responses, however, were produced at such high voltages that it would be difficult to mistake them for the reaction of a live pulp. The distribution of thresholds of live and necrotic pulps is shown in Table II. Of these teeth tested, 86.1 per cent responded to voltages between 1 and 20, whereas the response range for pulpless teeth was found mostly above 100 volts. In the two teeth classified as vital but giving a negative response, the pulp contained advanced pathologic processes

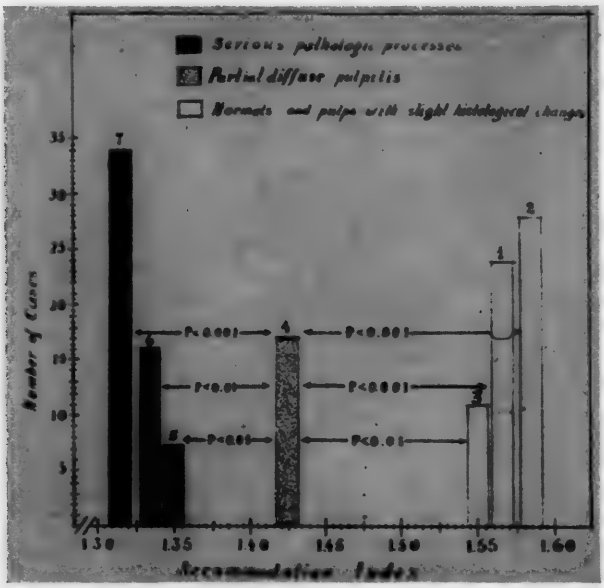

Fig. 11.—Graph showing statistical differences between groups. *1*, Normals. *2*, Reticular atrophies. *3*, Incipient inflammatory foci. *4*, Partial diffuse pulpitis. *5*, Hyperemias. *6*, Serious degenerative processes. *7*, Serious diffuse pulpitis.

TABLE II

RESPONSE RANGE OF LIVE AND NECROTIC PULPS

THRESHOLD (VOLTS)	VITAL TEETH		PULPLESS TEETH	
	NUMBER OF CASES	PER CENT	NUMBER OF CASES	PER CENT
1- 20	131	86.1	1	2.8
21- 50	4	2.6	0	–
51- 70	3	1.9	0	–
71-100	4	2.6	0	–
101-150	6	3.9	3	8.6
151-200	2	1.3	0	–
250 and over	0	–	3	8.6
Negative response	2	1.3	28	80.0
Total Cases	152		35	

which had destroyed a major part of it. The pulp channel of the only tooth with necrotic pulp giving a positive response below 20 volts was filled with a purulent liquid produced by an apical granuloma.

Repetitive Discharges.—In some teeth with pulpitis, autorhythmic discharges after the stimulation were found. These discharges indicated low accommodation; therefore, in cases in which the accommodation index could not be measured, the pulp responding in this manner was classified as abnormal.

Subjective Sensation.—The sensation was similar to a fine painless pricking.

Constancy of the Values in Time.—We repeated measurements in 17 normal and pathologic teeth, the time between the two controls being different in each case. The mean values of the accommodation indices obtained in the first control were statistically similar to those of the second control. The probability estimated for each case was very high and the percentage of variation of the values was low (Table III). The tooth resistance, which was different for every measurement, did not affect the accommodation index.

DISCUSSION

We have considered incipient foci as processes nearer to the normal than to the pathologic state. It has been demonstrated that the mere presence of shallow cavities[18] or cavity preparation[19] is enough to evoke tissue reactions in the pulp similar to those observed by us. This initial inflammatory reaction disappears once the tooth has been treated and filled.[20] For these reasons we have preferred to consider as correct the diagnosis indicating normality in these teeth. In some cases with a lowered accommodation index, a more advanced stage existed, although not so much as to be classified in Group II. It is clear that pulp disturbances present stages of transition that make it difficult to establish a strict classification in all cases. Perhaps it would be more accurate to say that, in the incipient inflammatory foci, the accommodation index could indicate correctly the most advanced processes of the group, and so our results may be also considered.

In partial diffuse pulpitis, a more generalized modification of pulpal tissue with alteration of the nerve response was observable. According to some authors,[20-21] these pulps may heal. We found phenomena of fibrosis circumscribing inflamed zones in regression. This led us to think that in such cases conservative action is advisable and, if possible, a repetition of the examination should be carried out after eliminating the caries.

We found hyperemia to be the main alteration of the pulp in only a few teeth. We believe, however, that such intense circulatory disorders, as those described elsewhere in the present paper, must have originated as a response to some chemical disturbance, possibly brought about by germ toxins, histamine, or vasodilator substances. We know, on the other hand, that acute circulatory disturbances threaten pulp vitality because of the great increment in tissular pressure produced by edema.[22] Pulpal tissue is enclosed in a hard chamber which limits its dilation, pressure rise being exerted against vessels which may collapse.[22]

Advanced histologic alterations observed in degenerative processes are likely related to the circulatory failure. The decrease of the blood supply produces in the tissues hypoxic states which tend to hyalinization.[23] In our opinion, these degenerative processes lead ultimately to the death of the pulp, and there is no guarantee that clinical symptoms will ever appear.

The pain and the decrease of nervous accommodation did not occur simultaneously in all the pathologic teeth. This is very strange, because nervous elements with a lowered accommodation are prone to discharge repetitively.[24] This autorhythmic activity should evoke pulpal pain, because it is assumed that teeth have only pain receptors.[25] Our results did not support this assumption. It is possible that receptors other than pain exist in the tooth. Pain receptors have a poor adaptation to prolonged stimuli, and a very high threshold,[25] unlike the characteristics found in the sensitive elements of the tooth. These nervous elements have a high accommodation[9] and therefore great adaptation.[27] The subjective sensation evoked by electric stimulation is painless[9-12] and the thresholds are remarkably low.[2] Loewenstein and Rathkamp[28] claimed an elevation of thresholds in pulpless teeth which they attributed to the possible existence of pressoreceptors inside the tooth. Specialized organized receptors have not been histologically shown in the pulp[29] and this leads us to think that the neurophysiologic phenomena observed in the tooth may correspond to sensitive elements in the dentine. The sensitive pathway of the dentine has not been physiologically studied yet.

Having in mind the above-mentioned facts, it is probable that the clinical symptoms in pathologic pulps are not due to a decrease in accommodation, as we have supposed in a previous paper,[9] but to the lowering of pain receptor threshold.

The measurement of accommodation is independent of the variations of tooth resistance (Table III). As the stimulation with the two currents is effected in one and the same spot and at practically the same time, the experimental conditions are the same for both measurements. If we measure an area of the tooth with high resistance, we shall need high voltages to find the threshold with both currents, but the relation between the voltages, i.e., the accommodation index, will be the same, or very nearly the same, as the one we would find if we made the measurement at a point with low resistance.

It can be concluded from our results that nervous accommodation varies correlatively with the physiopathologic changes of the pulp. This correlation does not occur with rheobase, which in some pulpal alterations increases and in other ones diminishes.[2]

The diagnosis of abnormal live pulps obtained by means of the method here presented makes the solution of numerous clinical problems possible, of which the following may be mentioned as being of major importance: facial neuralgias and infections of dental origin, advisable pulpectomies of teeth intended to support prosthetic appliances, diagnosis of teeth involved in cysts, apical granulomas, and fractures of the jaw.

We consider it extremely interesting to have been able to determine the gravity of the pulpal disturbances, not only from the morphologic point of view, but also from the physiologic one, as nervous accommodation is a sensitive index of the ionic[30-32] and circulatory[10, 11, 33] changes affecting the tissues where the nerve to be examined is located.

TABLE III
REPRODUCIBILITY OF THE ACCOMMODATION INDEX IN REPEATED CONTROLS

CASE NUMBER	TEETH NUMBER	DAYS BETWEEN CONTROLS	ACCOMMODATION INDICES		PROBABILITY P	PERCENTAGE OF VARIATION	TOOTH RESISTANCE (KILO-OHMS)	
			I_1	I_2			R_1	R_2
6	28	2	1.33	1.26	0.8-0.9	5.3	500	170
11	4	20	1.62	1.71	0.7-0.8	5.5	100	250
111	23	29	1.36	1.29	0.9	5.1	40	600
112	24	29	1.35	1.37	0.9	1.4	70	650
113	25	28	1.41	1.44	0.9	2.1	70	100
114	26	28	1.35	1.39	0.9	2.9	300	500
133	11	28	1.75	1.76	0.9	0.5	200	410
143	9	31	1.37	1.36	0.9	0.7	1000	1500
144	10	30	1.38	1.41	0.9	2.2	720	1900
165	23	32	1.45	1.44	0.9	0.7	900	2000
179	8	16	1.57	1.48	0.8-0.9	5.8	1000	1400
180	9	16	1.32	1.34	0.9	1.5	1000	1700
289	24	2	1.62	1.68	0.9	3.7	40	1400
331	9	8	1.40	1.41	0.9	0.7	2800	1000
343	9	2	1.20	1.40	0.6-0.7	14.3	1300	650
355	6	4	1.36	1.36	0.9	0.0	1650	2200
374	10	2	1.34	1.39	0.7-0.8	3.7	2250	1500
Number of Cases: 17						3.34 ± 3.43 Mean ± Standard deviation		

SUMMARY

Nervous accommodation of the sensitive elements of the pulp was determined in 152 teeth by measuring the accommodation index with 2 exponentially increasing currents of different rise-times.

Thirty-five teeth with pulpal necrosis were stimulated with the fastest exponential current.

Clinical symptoms were investigated to compare the efficiency of electrical and clinical methods in the diagnosis of pulp disturbances.

Results were checked by means of histologic examination of all the teeth studied.

Accommodation index showed a high correlation with the histologic pictures of the pulps, while clinical diagnosis showed a low correlation.

The accommodation indices of the normal and pathologic groups are statistically different, and decreased as disturbances increased.

Eighty per cent of teeth with pulpal necrosis did not react to electric stimuli, 17 per cent gave a response to more than 100 volts, and 2.8 per cent responded to fewer than 20 volts.

The stimulation with currents of exponential ascent was found to be painless, and the values reproducible and independent of tooth resistance.

Pulpal physiopathology was discussed.

The diagnosis of abnormal live pulps obtained with the accommodation index was considered of great importance to solve numerous clinical problems.

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THE EFFECT OF TOPICALLY APPLIED POTASSIUM FLUOROSTANNITE ON THE DENTAL CARIES EXPERIENCE IN CHILDREN

III. Results at the End of Three Years

CHARLES W. GISH, JOSEPH C. MUHLER, AND CHARLES L. HOWELL

Indiana State Board of Health, Indianapolis, Ind., and Indiana University, Bloomington, Ind.

IN A previous publication, the clinical procedure followed throughout this study as well as the effectiveness of a 4 per cent solution of potassium fluorostannite for reducing the incidence of dental caries in children at the end of 1 year was presented.[1] These data indicated 53.7 and 39.2 per cent less dental caries in the group receiving the potassium fluorostannite when compared to a group receiving distilled water, as evaluated by the DMFT and DMFS indices, respectively. After 2 years, there was no decrease in the effectiveness found at the end of 1 year.[2] In this paper, the clinical data will be presented comparing the effectiveness of an initial series of four topical applications of a 4 per cent solution of potassium fluorostannite to that of a similar number of applications of distilled water at the end of a 3-year study period.

EXPERIMENTAL

The method of dividing the children into their respective treatment groups, the technique of application, the clinical procedure, and the initial balance of the groups have been previously described.[1] Essentially, an initial treatment of 442 children, 6 through 16 years of age, with 4 applications of a 4 per cent aqueous solution of potassium fluorostannite was compared to 452 children with similar dental caries history and age range treated with 4 applications of distilled water.

DATA AND DISCUSSION

At the end of 2 and 3 years, respectively, there were 683 (364 control, 319 experimental) and 636 (342 control, 294 experimental) of the original 894 children available for re-examination. The balance of both the 2- and 3-year groups at the initial examination is good. There were initially an average of 5.5 and 5.6 DMFT, respectively, for the 2-year control and experimental groups; and 5.3 and 5.2 DMFT, respectively, for the 3-year control and experimental groups. The number of noncarious teeth at the initial examination of the 2-year group was 12.1 for the control and 11.5 for the potassium fluorostannite group. The number of noncarious teeth at the initial examination of the 3-year group was 12.2 for the control and 11.9 for the potassium fluorostannite group.

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The results of the four applications of the potassium fluorostannite on reducing dental caries in children, 1 and 2 years following the initial treatment, are shown in Table I and compared with the data obtained at the end of 3

TABLE I

A COMPARISON OF CARIES INCREMENTS AFTER ONE, TWO, AND THREE YEARS IN TWO GROUPS OF CHILDREN, ONE OF WHICH RECEIVED FOUR APPLICATIONS OF A 4 PER CENT SOLUTION OF POTASSIUM FLUOROSTANNITE AND THE OTHER GROUP FOUR APPLICATIONS OF DISTILLED WATER

		CONTROL	EXPERIMENTAL	PER CENT REDUCTION
1 Year	No. of Subjects	411	390	
	DMFT Increment	1.21	0.56	53.7 ($p = 0.000015$)
	DMFS Increment	2.42	1.47	39.2 ($p = 0.00014$)
2 Years	No. of Subjects	364	319	
	DMFT Increment	1.83	0.96	47.5 ($p = 0.00001$)
	DMFS Increment	4.42	2.72	38.5 ($p = 0.00006$)
3 Years	No. of Subjects	342	294	
	DMFT Increment	2.20	1.33	40 ($p = 0.00001$)
	DMFS Increment	4.58	2.74	41 ($p = 0.00001$)

years. The results 3 years following the initial treatment demonstrate a 40 per cent reduction in dental caries as evaluated by the DMFT index, and a 41 per cent reduction when the DMFS index is used. Both reductions are highly significant as indicated by their low probabilities.

It is not valid to compare these data with previously published data on stannous chlorofluoride or stannous fluoride. However, these data, as well as others, have suggested the superiority of the "tin" fluorides over sodium fluorides.[3, 4]

The major importance attached to these data is the duration of the effectiveness of this compound. Since its duration of effectiveness seems to be superior to stannous chlorofluoride,[5] additional study using this compound and related analogs with the "Single Application Technique"[4] seems indicated.

SUMMARY

Three years following the initial topical treatment of children with a 4 per cent potassium fluorostannite solution there were 40 and 41 per cent less dental caries as evaluated by the DMFT and DMFS indices, respectively, when compared to a similar control group which received distilled water. These data indicate that the effectiveness is still highly significant at the end of 3 years.

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COMPARATIVE ANALYSIS OF SALIVA IN PREGNANT AND NONPREGNANT WOMEN

I. Calcium and pH

S. LEONARD ROSENTHAL, ROBERT ROWEN, AND A. J. VAZAKAS

Temple University School of Dentistry, Philadelphia, Pa.

PREGNANCY may be accompanied by an increased incidence of dental caries unless unusually frequent prophylactic measures are instituted as a preventive. There is much diversity of opinion on the subject. Some authorities believe that pregnancy predisposes dental caries,[1, 2] while others feel it plays no role in this condition.[3, 4]

It has been suggested[5] that teeth decay during pregnancy because of changes in the secretions in the mouth. Since pregnancy is accompanied by metabolic changes, caries during this period may well be the result of a change in salivary composition and the ensuing modification of environment.

To determine what changes, if any, occur in the salivary composition during pregnancy, a series of salivary analyses has been undertaken. This is a report of the first series: a determination of the calicum content and pH of saliva of a group of pregnant women as compared with a nonpregnant group.

EXPERIMENTAL METHODS

For the purpose of determining the concentration of salivary calcium to be considered as normal for nonpregnant women, a group of 56 women, ranging in age from 17 to 27 years, was selected from the Temple University School of Oral Hygiene.

The saliva samples of the pregnant women were obtained from the out-patients of the obstetric clinics of 2 hospitals. The ages in the second group ranged from 14 to 38.

The women in both groups were all apparently normal healthy individuals. No physical examinations were given. However, care was taken to exclude women who had any recent history of illness.

All samples were collected in the morning, at least 1 hour after breakfast, and consisted of unstimulated saliva. For the collection of these samples, the subjects were kept as quiet as possible, usually in a seated position. They were instructed to allow the saliva to flow into the mouth as normally as possible, and to expectorate into large test tubes provided for the collection of the samples.

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Eight to ten milliliters of sample was collected in each case. The time required for the collection of samples was not recorded and rate of flow was not calculated. The pH of each sample was determined as soon as possible after collection.*

Of the methods available for the determination of total calcium, the method of Wainwright,[6] when modified by the inclusion of certain technics employed by Krasnow, Karshan, and Krejci,[7] was found best in our laboratory and was used throughout this study for the determination of total calcium.

A. *Preliminary Treatment of Samples.*—Becks and Wainwright[8] have shown that preliminary treatment of the saliva sample is necessary for the removal of substances which interfere with an accurate calcium determination. The preliminary treatment consists of centrifugation, for the removal of suspended material, and deproteinization, for the removal of salivary protein which, if present when the calcium is precipitated as the oxalate, prevents complete precipitation of the calcium.

Therefore, the saliva sample was centrifuged at 1,500 r. p. m. for 30 minutes, and 6.0 ml. of the supernatant fluid, measured carefully by means of a pipet, was transferred to a clean, dry 50 ml. Erlenmeyer flask. To this was added 6.0 ml. of 10 per cent trichloroacetic acid solution, accurately measured. The flask was swirled so that its contents were mixed thoroughly, and it was then allowed to stand for at least 10 minutes for complete deproteinization to occur. The precipitated proteins were removed by centrifuging the mixture for 30 minutes and the supernatant fluid was decanted and saved for the calcium determination.

B. *Precipitation of Calcium.*—Two 4.0 ml. aliquots of the deproteinized saliva, each corresponding to 2.0 ml. of original saliva, were measured by pipet into 2 clean, dry, 15 ml. graduated centrifuge tubes, the determination of calcium thus being run in duplicate. The remainder of the deproteinized solution was discarded. To each tube were added 1.0 ml. of 4 per cent ammonium oxalate and 2 drops of 0.04 per cent bromcresol purple. The reaction of each solution was adjusted to a pH of 5.9 by the addition of 1:1 NH_4OH, dropwise, until the color of the indicator changed from yellow to grayish-purple. About 3 or 4 drops of the ammonia solution usually sufficed. The tubes were swirled for thorough mixing to occur and then allowed to stand overnight for complete precipitation of calcium oxalate to take place.

The next day, the tubes were centrifuged for 30 minutes and the supernatant fluid was carefully poured off so as not to disturb the precipitate. The tubes were allowed to drain in an inverted position over filter paper for 5 minutes.

C. *Washing the Precipitate.*—The lip of each tube was dried, and to each tube were added 0.5 ml. of acetone† and 3 ml. of dilute ammonia (20 ml.

*A Model G Beckman pH meter was used.

†It was found[7] that a fine pellicle of calcium oxalate formed on the surface of the supernatant fluid after washing the precipitate of calcium oxalate with dilute ammonia. The surface pellicle was lost when the supernatant fluid was decanted, resulting in a somewhat lower calcium recovery. The addition of acetone to the calcium oxalate precipitate before washing it with dilute ammonia prevented pellicle formation by reducing surface tension.

of NH_4OH diluted with 980 ml. of distilled water). The dilute ammonia was added by means of a squeeze bottle so that the sides of the tubes were thoroughly rinsed. The tubes were swirled gently, then centrifuged for 30 minutes. (Less time spent on the centrifugation prevented the calcium oxalate from packing down strongly and crystals of it would pour off during the decantation.) The wash liquid was decanted, the tubes allowed to drain for 5 minutes in an inverted position over filter paper, and the lip of each tube was dried. The washing procedure with acetone and dilute ammonia was then repeated.

TABLE I

ANALYSIS OF TEST SOLUTION CONTAINING 0.2054 MG. OF Ca/2.0 ML.

AMOUNT OF SAMPLE USED (ML.)	CONCENTRATION OF CALCIUM FOUND (MG.)	DEVIATION (PER CENT)
2.0	0.2044	-0.5
2.0	0.1964	-4.3
2.0	0.1988	-3.2
Average deviation		-2.7

TABLE II

CALCIUM CONTENT AND pH OF UNSTIMULATED SALIVA OF 52 NONPREGNANT WOMEN

SAMPLE NUMBER*	WOMAN'S AGE	pH OF SAMPLE†	TOTAL CALCIUM (MG./100 ML.)	SAMPLE NUMBER*	WOMAN'S AGE	pH OF SAMPLE†	TOTAL CALCIUM (MG./100 ML.)
1	18	5.8	4.7	30	18	7.3	4.7
2	18	7.1	4.8	31	18	6.7	4.1
3	18	5.9	7.3	32	19	6.9	5.9
4	18	7.1	6.0	33	18	6.7	4.7
5	18	7.2	3.8	34	18	6.9	5.3
6	17	7.0	4.8	35	19	7.3	4.4
7	17	7.0	6.1	36	20	6.8	5.6
8	18	7.0	5.0	37	19	6.6	4.7
9	18	7.4	4.5	38	18	7.2	6.8
10	20	7.0	5.0	39	19	7.3	5.0
11	18	7.3	5.1	40	19	7.2	4.5
12	18	6.8	4.6	41	19	7.1	5.8
13	19	6.9	7.0	43	19	7.3	5.2
14	19	7.1	7.0	45	19	7.2	5.7
15	19	6.9	4.8	46	19	7.4	6.6
16	25	6.5	5.4	47	18	7.3	5.4
17	18	6.7	5.7	48	19	7.4	4.2
18	19	7.0	4.4	50	18	6.8	5.8
19	19	7.3	4.1	51	19	7.3	5.5
20	18	7.0	5.1	52	27	7.2	4.6
21	19	7.0	4.4	53	18	7.2	5.1
22	20	6.7	6.0	54	18	6.6	6.3
23	21	7.2	5.8	55	20	7.1	5.2
24	19	6.3	5.9	56	19	7.0	5.1
26	23	6.6	5.2				
27	18	7.2	6.3	Arithmetic Mean		7.0	5.3
28	18	7.1	5.2	Standard Deviation		0.3	0.8
29	19	7.3	6.5				

*Samples 25, 42, 44, and 49 rejected as deviating from normal because of odd color and/or foul odor. Calcium content and pH were not determined.

†All pH readings taken with Model G Beckman pH meter.

TABLE III
CALCIUM CONTENT AND pH OF UNSTIMULATED SALIVA OF 61 PREGNANT WOMEN

SAMPLE NUMBER	WOMAN'S AGE	NO. OF MONTHS PREGNANT	PH OF SAMPLE*	TOTAL CALCIUM (MG./100 ML.)	SAMPLE NUMBER	WOMAN'S AGE	NO. OF MONTHS PREGNANT	PH OF SAMPLE*	TOTAL CALCIUM (MG./100 ML.)
P-1	31	2.5	6.9	6.2	P-33	35	8	7.0†	4.7
P-2	34	4	6.9	5.1	P-34	33	5	6.0†	‡
P-3	22	7	6.8	5.3	P-35	36	8.5	6.3	5.2
P-4	27	8.5	6.4	6.1	P-36	23	8.5	6.6	5.0
P-5	23	4.5	7.2	4.2	P-37	29	6	6.4	‡
P-6	34	8.5	6.1	7.4	P-38	24	8.5	6.7	5.1
P-7	22	7	6.9	5.2	P-39	28	6	5.4	5.8
P-8	26	6.5	6.6	6.0	P-40	22	3	6.1	4.1
P-9	32	8.5	5.1	5.9	P-41	27	6	‡	‡
P-10	27	8	6.4	7.3	P-42	18	5	7.4	‡
P-11	19	7	5.9	7.2	P-43	21	7	‡	‡
P-12	22	8	6.6	6.7	P-44	30	7	6.8	6.0
P-13	21	7	6.0†	5.8	P-45	18	6	5.9	‡
P-14	38	6	6.0†	5.0	P-46	24	7	6.4	5.6
P-15	22	7	7.5†	3.8	P-47	20	7	6.6	4.3
P-16	29	8	6.0†	5.1	P-48	22	8.5	6.3	5.0
P-17	16	7	7.5†	4.6	P-49	21	7	6.5	4.0
P-18	28	8.5	7.0†	4.6	P-50	21	8.5	6.2	‡
P-19	27	8.5	6.0†	5.0	P-51	19	6	5.8	6.5
P-20	29	8	6.0†	7.7	P-52	21	8.5	6.7	5.2
P-21	16	5	6.0†	4.1	P-53	21	8	6.6	3.9
P-22	18	4	5.5†	6.0	P-54	20	4	7.0	4.2
P-23	18	6	6.0†	4.3	P-55	33	4	6.7	5.8
P-24	23	7	6.0†	4.4	P-56	20	4	6.6	3.9
P-25	28	6	5.5†	3.2	P-57	26	4	6.4	4.5
P-26	23	4	7.0†	3.6	P-58	26	3.5	7.0	4.9
P-27	26	6	5.0†	5.9	P-59	33	5.5	6.6	3.8
P-28	30	2	5.0†	6.6	P-60	14	3	7.1	5.0
P-29	35	7	7.5†	3.6	P-61	16	7	7.0	5.2
P-30	37	7	6.0†	‡					
P-31	23	8.5	6.0†	4.5	Arithmetic Mean			6.5§	5.2
P-32	18	8	5.5†	7.3	Standard Deviation			0.5§	1.1

*All pH readings, unless otherwise indicated, taken with Model G Beckman pH meter.
†Approximate pH readings, measured with Nitrazine paper.
‡Not enough sample received for determination.
§Based only upon the 37 pH readings taken with the Model G Beckman pH meter.

D. *Total Calcium Determination.*—When the tubes had drained following the second washing of the calcium oxalate, and the lip of each tube had been dried, 2.0 ml. of 1.0 N H_2SO_4 was added to each tube. The sides of each tube were rinsed down with about 2 ml. of distilled water, and the tubes were placed in a water bath at 80° C. When all of the precipitate in the tubes had dissolved, the solutions were titrated with a 0.01 N $KMnO_4$ solution delivered by means of a microburet of 5.0 ml. capacity, graduated in hundredths of a milliliter. A blank, consisting of 2.0 ml. of 1.0 N H_2SO_4, was also titrated. The exact normality of the 0.01 N $KMnO_4$ solution was determined on each day of use by standardization against a standard solution of sodium oxalate.[9]

The content of calcium, expressed as milligrams per 100 ml. of saliva, was computed according to the following formula:

$$x(y\text{-}z)\ (0.02004)\ (1000)\ (50) = \text{mg. of calcium/100 ml. sample.}$$

x = normality of $KMnO_4$.
y = number of ml. of $KMnO_4$ used in titration of sample.
z = number of ml. of $KMnO_4$ used in titration of blank.
0.02004 = millieqivalent weight of calcium.

E. *Evaluation of Method.*—To investigate the possibility of loss of calcium during the several steps of the analysis, a series of determinations was run on a calcium solution of known concentration. An accurately weighed amount of calcium carbonate, Baker's analyzed reagent, which had been dried overnight at 125° C. prior to being weighed, was made up to a known volume with distilled water and with 1 ml. of HCl to effect solution. This calcium standard contained 0.2045 mg. per 2.0 ml. of solution. Three 2.0 ml. samples of this solution were analyzed for calcium by the method described previously.

This method of analysis gave a deviation from the actual amount of calcium present of from –0.5 to –4.3 per cent with an average deviation of –2.7 per cent (Table I). This loss probably occurred during the washing procedure.

RESULTS AND CONCLUSIONS

The data for the individual cases studied during this investigation are given in Tables II and III. There appeared to be no statistical difference between the calcium content of the saliva of nonpregnant women and that of pregnant women, the mean concentration being 5.3 mg. per cent and 5.2 mg. per cent, respectively.

A significant difference, however, appeared with respect to the pH values found in the 2 groups. The mean pH value for the saliva of nonpregnant women was 7.0; that for the saliva of pregnant women was 6.5.

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THE EFFECT OF HEAT TREATMENT VARIABLES ON THE MICROSTRUCTURE AND HARDNESS OF A CAST DENTAL GOLD ALLOY

ANTHONY K. KAIRES,* AND JOSEPH C. THOMPSON**

U. S. Naval Station, San Francisco, Calif., and U. S. Navy Electronics Laboratory, San Diego, Calif.

A METALLOGRAPHIC investigation of a type IV, extrahard, dental gold alloy[1] was undertaken in order to study the relationship between microstructure and hardness of a cast gold alloy which had been heat treated under various conditions.

The qualitative and quantitative composition of the gold† used in this study was given as gold, 71.1 per cent; palladium, 4.0 per cent; silver, 9.0 per cent; copper, 15.4 per cent; and zinc, 0.5 per cent.[2] The constituents of this alloy have been investigated as (1) binary alloys (Ag-Au,[3] Ag-Cu,[4] Ag-Pd,[5] Ag-Zn,[6] Au-Cu,[7] Au-Pd,[8] Au-Zn,[9] Cu-Pd,[10] Cu-Zn,[11]), as (2) ternary alloys (Ag-Au-Pd,[12] Ag-Cu-Pd,[13] Ag-Cu-Zn,[14] Au-Ag-Cu[15]), and a (3) quaternary alloy (Au-Pd-Cu-Ag[16]).

The concept of intermetallic transformations in solid solutions, and the phenomenon of precipitation hardening, with theoretical explanations pertaining thereto, are well presented in the metallurgical literature.[17-21]

In the solidification of precipitation-hardenable alloys from a molten state there is an interrelation of time, temperature, and transformation. The Gibbs phase rule[22] presents this interrelation diagrammatically in the form of equilibrium or phase diagrams.[3-14] Its statements, however, are restricted to the number and compositions of thermodynamic phases that are in equilibrium. Under conditions of nonequilibrium, as in rapid cooling, variations in the composition of a solid solution are possible. It has been reported that no serious segregation of this type occurs during routine dental casting operations.[23] Crystal imperfections produced during solidification, other than shrinkage porosities and gas inclusions, have been attributed to unsoundness in grain boundaries, and unsoundness between dendritic branches.[24] Variations in mechanical properties in cast dental alloys have been attributed to variations of

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*U. S. Naval Station, Navy 961, FPO, San Francisco, Calif.

**Metallurgist, U. S. Navy Electronics Laboratory, San Diego, Calif.

†Manufactured by the Spyco Smelting and Refining Company, Minneapolis, Minn.

grain sizes, shrinkage porosities, impurities, gas inclusions, segregations, coring, and precipitations.[24-27] These variations account for the scatter of physical values obtained during testing procedures. The physical property of hardness has been used to indicate and study intermediate phases in alloy systems.[28] Because of observed correlations, it also has been employed as a clue to other physical properties, especially tensile yield point. Ultimate tensile strength may be approximated from a hardness number and assumptions may be made from this tensile strength concerning the yield point of the material.

There are many interrelated factors that must be considered in the successful casting of partial denture gold alloy[31, 32] which, if overlooked or carelessly handled, could adversely affect the desired characteristics of a casting. Subsequent heat treatments are designed to produce desired mechanical properties in a casting, but they cannot be expected to rectify the results of careless, irresponsible original work. The treatments consist of a "combination of heating and cooling operations, timed and applied to a metal or alloy in the solid state in a way that will produce desired properties."[33]

The heat treatment widely used in dental annealing operations, and in this study, is placing the casting in a preheated furnace at 1,290° F. for 10 minutes and quenching in water at room temperature.[1, 34-36] Annealing decreases hardness and strength and usually increases ductility. The microstructure is altered to a relatively homogeneous solid solution with a reduction of segregations. The precise mechanism is obscure, but it is believed to be brought about by means of some structural alteration by slow diffusion.

Hardening of gold alloys can be accomplished by one or two general methods, each of which has as its purpose the control of intermetallic transformations in solid solutions. A hardening treatment increases hardness and strength, and decreases ductility.

PREPARATION OF SPECIMENS

The cast gold alloy used in this study was heat treated by the following methods.

I. *Variable Time-Temperature Method.*—

1. A series of four castings, with ten test specimens in each, was made under uniform conditions (Fig. 1). The test specimens were prepared with due consideration to each of the following factors: uniformity in the shape and size of the wax patterns; the positioning of the wax patterns in the casting ring; the lengths, gauge* (0.06 inch), configuration, place and manner of attachment of the sprues; and uniformity of investing, burn-out, and casting procedures. The four castings were made under identical conditions using an electrically controlled casting unit.† Each casting was made with equal amounts of new gold alloy, at the recommended temperature, and with the same force to throw the metal into the mold. The castings were permitted to bench cool for 5, 10, 15, or 20 minutes, before quenching in water. They were then cleaned, pickled, and numbered for identification purposes.

*No. 14 gauge, Brown and Sharpe (gauge table).
†Thermotrol, J. F. Jelenko & Co., Inc., New York, N. Y.

Each casting was equally divided into two groups of five specimens each, with each of the specimens related to its original position in the radius of the ring. The 20 specimens comprising Group A were used to study the effect of bench cooling the castings within the mold for variable time intervals before quenching in water. The remaining specimens, Group B, were reserved for other methods of heat treatment.

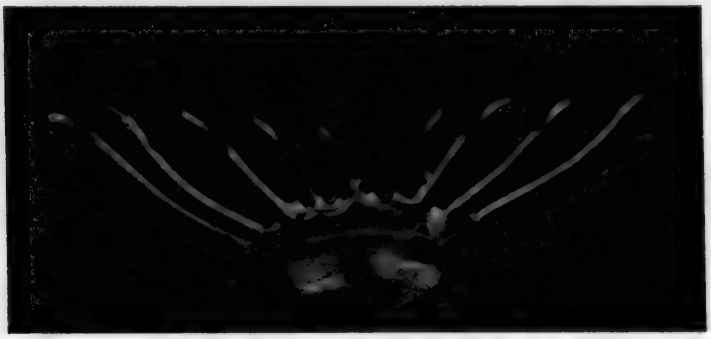

Fig. 1.—A test casting to show the position, length, and manner of attachment of the sprues to the test specimens.

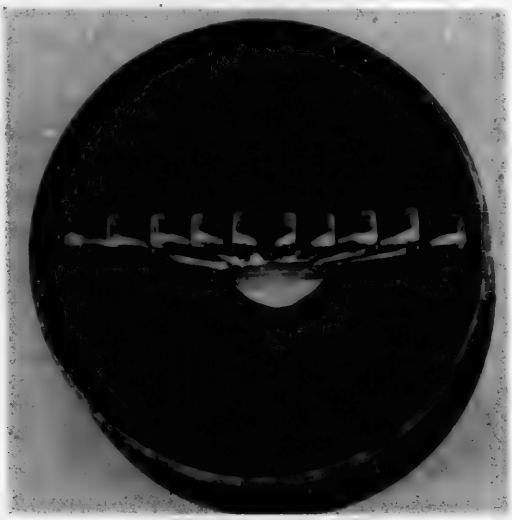

Fig. 2.—One of the castings repositioned on a sprue former to show the relation of the specimens to each other and to the casting ring.

The Group A specimens were removed and potted in metallurgical hard Bakelite. The preparation of metallographic specimens was made in accordance with the recommendations contained in ASTM designation, E3-46T.[37] The surfaces were etched with a freshly prepared solution of equal parts of potassium cyanide and ammonium persulfate.[38] A Tukon tester, with a Knoop indenter, was used to determine the Knoop hardness number for each specimen. Three

indentations were made, using a load of 1.0 Kg. for 20 seconds. These indentations were located in identical positions on each specimen and spaced 15 mm. apart. The length of the long diagonal of each diamond-shaped indentation was measured in filar units and then converted to microns by the factor of 0.7062. The Knoop hardness number was determined from appropriate tables provided by the manufacturer. The results of this method of heat treating are presented in Table I.

TABLE I
VARIABLE TIME-TEMPERATURE HEAT TREATMENT (1)

Specimen-ring distance (mm.)	36	28	20	11	2
Sprue length (mm).	11	14	18	25	34
BENCH COOLING PERIOD	KNOOP HARDNESS NUMBERS (MEAN VALUES)				
5 minutes	152	143	151	143	140
10 minutes	154	149	153	136	154
15 minutes	143	149	156	183	217
20 minutes	230	222	209	209	222

Each Group A specimen was examined under magnification, × 100, on a metallograph. After an exploratory examination, a field was selected that included the indentations for hardness. The field was photographed on a metallographic plate with an average exposure time of 2 seconds (Figs. 3-6). Note characteristics of the structure of the fully annealed specimens shown in Figs. 3*A* and 6*A* as compared to the hardened dendritic structure shown in Figs. 3*E* and 6*E*. In addition, appropriate adjustments were made on the metallograph to photograph* a select field at a magnification of 50 times, using polarized light. The average exposure time was 4 seconds. In the metallographic examination, particular attention was made relative to grain size, the presence or absence of structural defects, and the degree of dendritic segregation. Phase contrast metallographic examinations were also made on select specimens.

2. All the specimens in Group B were annealed by heat soaking in a preheated furnace at 1,290° F. for 10 minutes and quenching in water. The specimens originally located closest to the casting ring, and those located innermost to the center were potted in metallurgical hard Bakelite, polished, etched, and tested for hardness. The remaining specimens were placed in a furnace preheated to 850° F. and gradually reduced to 450° F. in 30 minutes. They were then removed and quenched in water at room temperature. These specimens were similarly potted, polished, etched, and tested for hardness. The results using this method of heat treatment are presented in Table II. The specimens were examined on the metallograph and photographed (Fig. 7). All the specimens were removed from their original potted condition to be used in other methods of heat treating.

3. Six specimens were placed in a preheated furnace at 1,290° F. for 10 minutes. Three specimens were removed from the furnace and allowed to bench cool. The remaining 3 specimens were permitted to cool slowly in the furnace with the furnace door open and the control switch in the "off" position.

*Kodachrome Professional Film (KA 135), Type A, was used.

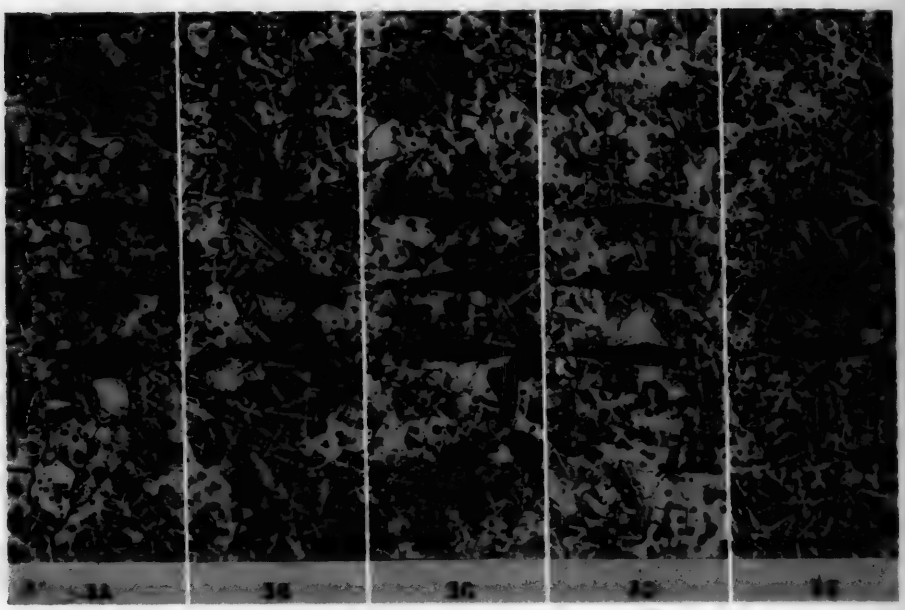

Fig. 3.—The photomicrographs of 5 specimens (magnification ×100) after the mold had bench cooled 5 minutes and was quenched in water. *A*, Innermost specimen. *B*, *C*, *D*, Intermediate specimens. *E*, Specimen closest to the casting ring.

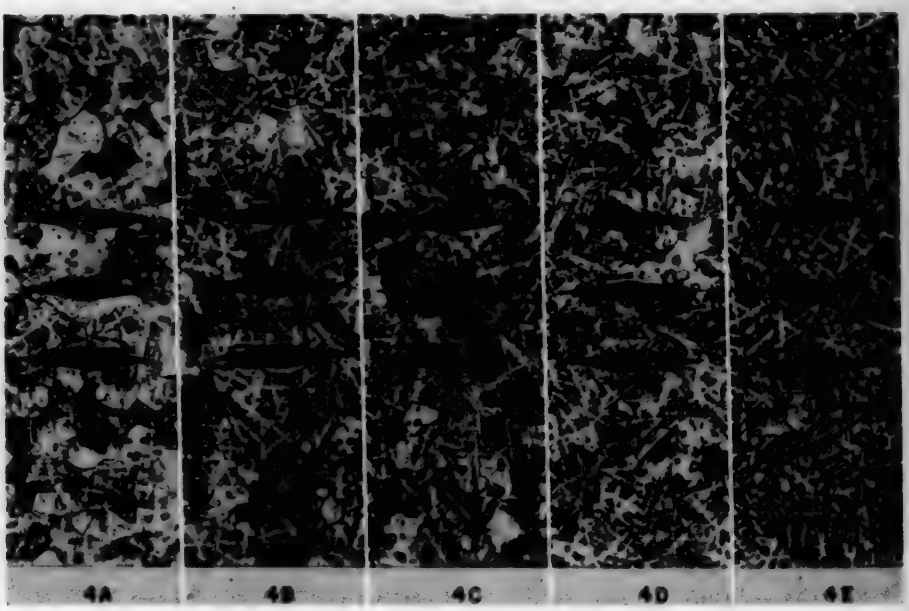

Fig. 4.—The same as Fig. 3, except that the mold was bench cooled 10 minutes.

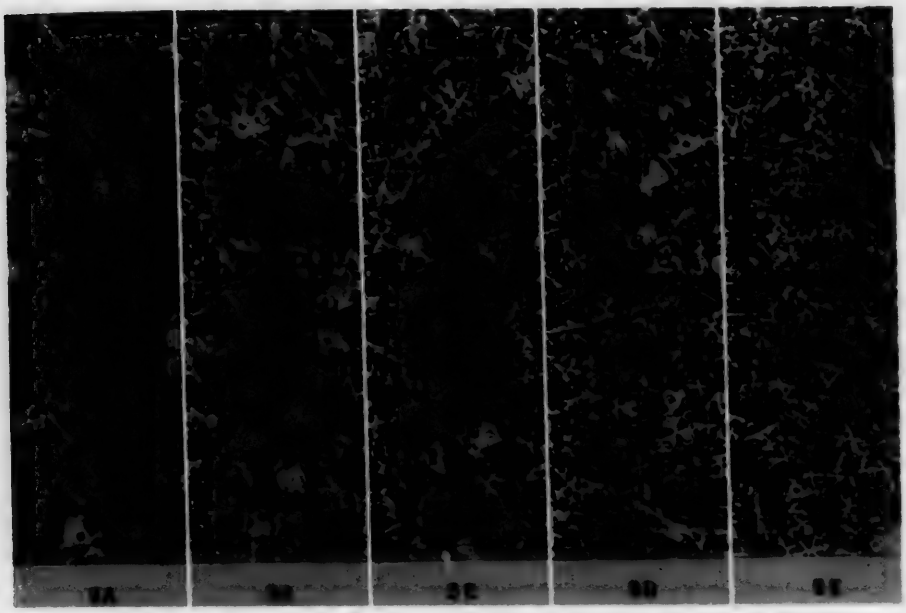

Fig. 5.—The same as Fig. 3, except that the mold was bench cooled 15 minutes.

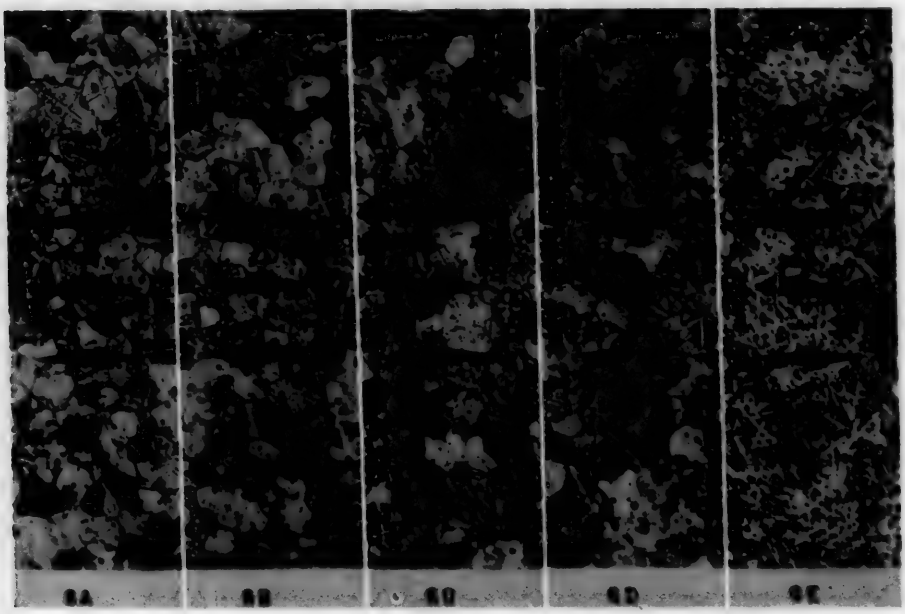

Fig. 6.—The same as Fig. 3, except that the mold was bench cooled 20 minutes.

Three additional specimens were heated to redness over the open flame of a Bunsen burner and permitted to bench cool. All 9 specimens were then potted, polished, etched, tested for hardness, and photographed. The results of this method of heat treatment are presented in Table III.

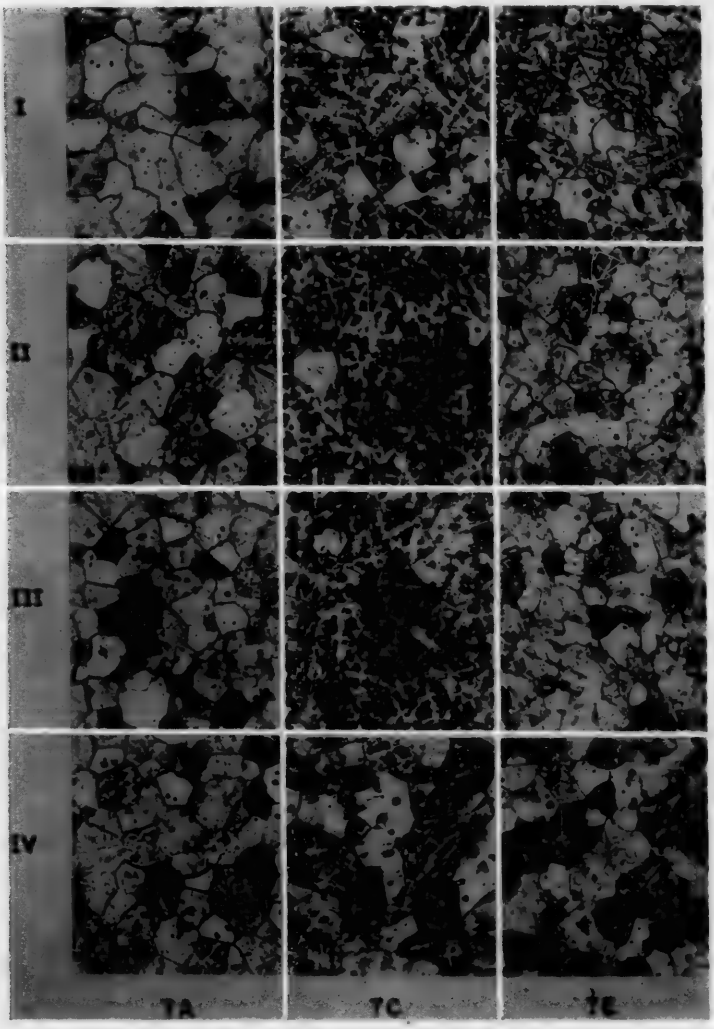

Fig. 7.—Photomicrographs of Group B specimens (magnification ×100).
I, Mold was bench cooled 5 minutes. *II*, Mold was bench cooled 10 minutes. *III*, Mold was bench cooled 15 minutes. *IV*, Mold was bench cooled 20 minutes.
A, Annealed innermost specimens. *C*, Hardened, intermediate specimens (850° F. to 450° F. in 30 minutes). *E*, Annealed specimens closest to the casting ring.

II. *Constant Time-Temperature Method.*—

1. Ten specimens were annealed. Nine of the specimens, in 3 groups of 3, were heat soaked in a preheated furnace at a constant temperature of 450° F., for 5, 10, and 20 minutes, respectively, and quenched in water at room temperature.

TABLE II
VARIABLE TIME-TEMPERATURE HEAT TREATMENT (2)

Specimen-ring distance (mm.)	36	28	20	11	2
Sprue length (mm.)	11	14	18	25	34

	KNOOP HARDNESS NUMBERS (MEAN VALUES)				
BENCH COOLING PERIOD	ANNEALED	ANNEALED AND HARDENED (850° F. TO 450° F. IN 30 MINUTES)			ANNEALED
5 minutes	211	249	239	260	182
10 minutes	164	239	260	243	190
15 minutes	192	247	251	226	191
20 minutes	200	224	235	237	173

TABLE III
VARIABLE TIME-TEMPERATURE HEAT TREATMENT (3)

MODE OF HEAT SOAKING	ANNEALING	HARDENING TREATMENT		METHOD OF QUENCHING	KNOOP HARDNESS NUMBERS (MEAN VALUES)
		TEMPERATURE	TIME		
Preheated furnace	1,290° F. for 10 minutes	1,290° F. to room temp.	20 min.	Air cooled	159 138 152
			3 hr.	Open furnace	249 219 251
Bunsen burner	Red hot	Red hot to room temp.	20 min.	Air cooled	154 156 156

All the specimens were then potted, polished, etched, tested for hardness, and photographed. The results of this method of heat treatment are presented in Table IV.

TABLE IV
CONSTANT TIME-TEMPERATURE HEAT TREATMENT

ANNEALING	HARDENING TREATMENT		METHOD OF QUENCHING	KNOOP HARDNESS NUMBERS (MEAN VALUES)
	TEMPERATURE	TIME		
1,290° F. for 10 minutes Quenched in water	Furnace 450° F.	5 min.	Room temperature water	239 243 237
		10 min.		243 237 243
		20 min.		243 249 253
				203
1,290° F. for 10 minutes Quenched in water	Salt Bath 450° F.	5 min.	Room temperature water	247 253 253
		10 min.		247 249 249
		20 min.		264 243 258
				198

2. Ten specimens were annealed. Nine of the specimens, in 3 groups of 3, were heat soaked in a salt bath consisting of a mixture of potassium nitrate and

sodium nitrate held at a constant temperature of 450° F., for 5, 10, and 20 minutes, respectively, and quenched in water at room temperature. The results are presented in Table IV.

PHOTOMICROGRAPHIC EXAMINATION

Black and white photomicrographs were taken of all specimens at 100 times magnification (Figs. 3-9). The photomicrographs of the Group A and B specimens were arranged on a table in the same relative positions as the specimens originally were in the casting ring. Four series of 5 photomicrographs were thereby conveniently laid out for comparative study. Differences could be studied not only in relation to the same series but also in relation to the other quenches.

Individual Kodachrome color transparencies of the test specimens at a magnification of 50 times were also obtained, and were projected onto a beaded screen. This method of photomicrographic examination provided an excellent means to study the microstructure of the specimens.

Fig. 8. Fig. 9.

Fig. 8.—Photomicrograph of specimen hardened by the constant time-temperature method, furnace at 450° F. for 10 minutes (magnification ×100).

Fig. 9.—Photomicrograph of specimen hardened by the constant time-temperature method, salt bath at 450° F. for 5 minutes (magnification ×100).

POLISHING TEMPERATURES

In order to determine the amount of heat that was generated during the polishing of routine cast partial dentures, a copper vs. constant thermocouple was used in conjunction with a Leeds and Northrup potentiometer. The thermocouple was placed opposite the area in which the grinding was to be performed, and made secure with adhesive tape. A minimum of 6 readings was made for each operation during the grinding and polishing procedures. The gauge* of the sprues was 0.06 inch. The dental lathe was operated at high speed (3,400 r.p.m.). Temperatures were recorded during the cutting and the subsequent reduction of the sprues, and the polishing of the clasps. The findings are presented in Table V.

RESULTS

Dendritic structure was found in all specimens in Group A; however, the amount of this structure seemed to be a function of the distance from the casting ring. This relationship is borne out in Figs. 3-6 where the *E* photomicrograph

*No. 14 gauge, Brown and Sharpe (gauge table).

TABLE V
GRINDING AND POLISHING TEMPERATURES

OPERATION	TEMPERATURE RANGE (DEGREES F.)	AVERAGE (DEGREES F.)
Sprue Cuts		
Disk, abrasive, silicon carbide, ⅞ inch, double cutting	125 – 185	154
Disk, abrasive, silicon carbide, ⅞ inch, safesided	130 – 145	139
Sprue Reduction		
Wheel, abrasive, silicon carbide, unmounted, assorted	118 – 153	144
Wheel, abrasive, silicon carbide, mounted, assorted	90 – 110	105
Wheel, impregnated rubber, fine abrasive, ⅛" × ⅞"	130 – 150	133
Wheel, abrasive, rubber, sulci-wheel shape, 0.625 in. diameter, Burlew type	100 – 135	126
Clasps		
Wheel, abrasive, silicon carbide, cylinder and tapered cylinder	80 – 100	94

shows the condition nearest the ring. The variation in grain structure shown in these figures seems to have had very little influence upon the hardness of the specimen. No explanation for this phenomenon was apparent, and the scope of this investigation did not permit research in this area.

Table I shows that the specimen hardnesses were very erratic below the 15-minute period before quenching, but, at 20 minutes, all parts of the casting had very nearly the same hardness.

An interesting phenomenon in this group was the way in which the 15-minute specimen differed from the others. In comparing Figs. 3-6 it is noticed that Fig. 5 seems to hold to the dendritic structure regardless of distance from the casting ring. This uniqueness is again observed in Table I where it can be seen that the relative hardnesses varied almost directly with the distance from the casting ring. Further investigation might prove a critical point in the structural transformation near this particular time lapse before quenching.

ASTM grain-size plates[39] were used to designate the grain sizes found in these specimens.

The Group A specimens were subjected to an annealing process. The inner and outer specimens, relative to their mold position, were prepared for metallographic examination while the others were heat treated, as shown in Table II, before such preparation. Fig. 7 shows the effect of these treatments. As shown in Fig. 7, *E*, the grain structure varied considerably depending upon the bench time allowed before quenching. The 10- and 15-minute specimen (Fig. 7, *E*-II and 7, *E*-III, respectively) showed homogeneous structures having a grain size of No. 5.5. The 5- and 20-minute specimens were somewhat less homogeneous with an increased grain size of No. 4.5 (Fig. 7, *E*-I and 7, *E*-IV).

The innermost annealed specimens all exhibited a relatively homogeneous microstructure with a grain size of No. 4.5 (Fig. 7, *A*). Generally, it can be stated that in the annealed specimens, the grain sizes ranged from No. 4 to

5.5 with a preponderance of No. 4.5. It was impossible to determine the hardness of a specific specimen in terms of microstructure alone. It must be added that only one set of castings, of one alloy, was used in this study.

Results of the individual heat treatment methods follow.

I. *Variable Time-Temperature Method.*—

1. The hardness results of heat treating by quenching in water, after the mold has been bench cooled a given period of time, are presented in Table I. Photomicrographic evidence of microstructural variations are presented (Figs. 3-6). On the basis of the observed findings, it appears that this method of heat treatment is of questionable value other than to obtain the optimal conditions for subsequent heat treatments in order to modify existing segregations. A partial denture designed for casting is ordinarily of an irregular shape, and of variable size and bulk. In addition, its positioning in the casting ring, and the factors of number, length, and manner of attachment of the sprues, would all contribute to a nonuniform casting. Subsequent heat treatments are indicated to obtain greater uniformity.

2. The results of annealing the outer and inner specimens, and the hardening of the intermediate specimens are presented in Table II. Photomicrographic evidence of variations in annealing and hardening by this method are presented (Fig. 7). This method of heat treatment did not produce the most desirable microstructural form, as evidenced by the segregations found in the hardened state (Fig. 7). Annealing time and/or temperature appeared to be inadequate for those specimens previously conditioned by excessive dendritic segregations.

3. The hardness results by the method of heat treatment involving annealing at 1,290° F. for 10 minutes, and bench cooling, are presented in Table III. The rate of cooling through the critical range of hardening temperatures determined the degree of hardening; rapid cooling did not produce a hardening effect, but slow cooling did produce this effect. The microstructure exhibited considerable evidence of segregations. This method of heat treating is not recommended because of its unpredictable results, which were dependent upon variable thermal environmental conditions.

II. *Constant Time-Temperature Method.*—

Those specimens which were heat soaked in either a preheated furnace or a salt bath, at 450° F., for 5, 10, or 20 minutes, and subsequently quenched in water at room temperature, produced a microstructure similar to that found in an annealed condition, with an ASTM grain size of No. 4.5 (Figs. 8 and 9). The specimens examined were found to be relatively free of segregations. This method of heat treating is preferred because of its simplicity and the uniformity of its results. The hardness results using this method of heat treating are presented in Table IV.

CONCLUSIONS

A cast dental gold alloy, when put through a hardening treatment after annealing, tended toward a dendritic structure except when using the constant

time-temperature procedure (Fig. 7). This structure, however, had little or no influence on the hardness of the material (Compare Fig. 6 and Table I).

Table II would seem to show that, in order to obtain a harder casting, the quenching should be done within 5 minutes after withdrawal from the furnace, and the casting should be heat treated as shown in the table. This process yielded a greater hardness than the other procedures but tended toward greater variation than the constant time-temperature treatment, shown in Table IV, which produced a casting with a more homogeneous grain structure and with consistent hardness values throughout.

Generation of heat during grinding and polishing the cast partial denture elevated the local temperature of the material to something less than 200° F. (see Table V). This heating was of short duration and was not great enough to produce any transformation of the solid solution. Such short periods at these temperatures were considered harmless.

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PRETREATMENT OF TEETH WITH SODIUM FLUORIDE PRIOR TO STANNOUS FLUORIDE APPLICATION IN CHILDREN

H. ABDUL-GHAFFAR, AND JOSEPH C. MUHLER

Department of Biochemistry, Indiana University, Indiana University Medical Center, Indianapolis, Ind.

IT HAS generally been assumed that the anticariogenic effect of topically applied fluorides decreases with time, and, in order to maintain their effectiveness, a series of treatments needs to be repeated about every 3 years. Similarly, the repeated series of treatments is required in order to provide protection to the newly erupted teeth which did not receive the initial fluoride therapy. In order to provide protection at a higher level, and to simplify the four-treatment technic required for sodium fluoride, the single stannous fluoride technic was designed. Such studies have shown the superiority of the single application technic over the series of four applications of sodium fluoride.[1-3] Jordon and his co-workers[4, 5] have confirmed the anticariogenicity of the single application technic. In order to determine if one could develop conditions favorable to an even higher degree of protection, laboratory studies were designed to investigate those conditions which seemed optimal for the use of highly concentrated solutions of stannous fluoride. As a result of these studies, it was found that the pretreatment of rat molars with a 4 per cent solution of sodium fluoride, followed by a single application of stannous fluoride, was not only more effective against acid decalcification, but its duration of effectiveness was more prolonged.[6] Since any such postulate obtained in animals requires evaluation in the human being, this study was conducted to test this in vitro finding.

EXPERIMENTAL METHODS

Approximately 600 children from Spencer, Indiana, 6 to 15 years of age, were divided into three groups according to their past dental caries susceptibility.[7] Group I received a single application of a 4 per cent aqueous sodium fluoride solution and a single application of an 8 per cent stannous solution the following day. Group II received two applications of an 8 per cent stannous fluoride solution, the second treatment applied the following day. Group III received no topical therapy and served as controls. All three groups received a thorough dental prophylaxis, and the first fluoride application was given immediately thereafter by methods which have previously been described in detail.[8] After 1 year the children were re-examined by the same dentist, and all precautions for eliminating examiner bias, as previously described[9] were followed.

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DATA AND DISCUSSION

The number of subjects, their mean age, the mean number of permanent and permanent-sound teeth, and their mean caries history, as expressed by the DMFT and DMFS indices at the initial examination, are seen in Table I.

TABLE I
NUMBER OF SUBJECTS AND THEIR PREVIOUS DENTAL CARIES HISTORY

GROUP	NO. OF SUBJECTS	MEAN AGE (YR. AND MO.)	MEAN TOTAL NO. PERMANENT TEETH AT INITIAL EXAM.	MEAN TOTAL NO. SOUND PERMANENT TEETH AT INITIAL EXAM.	MEAN DMFT AT INITIAL EXAM.	MEAN DMFS AT INITIAL EXAM.
$NaF + SnF_2$	199	13.34	18.47	12.95	5.51	8.25
SnF_2	211	13.22	17.91	12.46	5.42	8.54
Control	192	12.70	18.40	12.94	5.48	8.49

The number of subjects available for re-examination at the end of the year, along with their appropriate dental caries history, is seen in Table II. These data show that only minor differences, as evaluated by the DMFT and DMFS indices, were found between the original sample and those available for re-examination.

TABLE II
DENTAL CARIES INCREMENTS IN CHILDREN FINISHING THE STUDY PERIOD

GROUP	NO. OF SUBJECTS	INITIAL EXAMINATION		SECOND EXAMINATION		INCREMENTS		% REDUCTION	
		MEAN DMFT	MEAN DMFS	MEAN DMFT	MEAN DMFS	DMFT	DMFS	TEETH	SURFACES
$NaF + SnF_2$	182	5.54	8.34	5.52	8.90	−0.02	0.56	100	60.6
SnF_2	185	5.38	8.58	5.35	8.89	−0.03	0.31	100	78.2
Control	169	5.48	8.63	6.12	10.05	0.54	1.42	—	—

The effect of the various fluoride treatments on the incidence of dental caries is also seen in Table II. These data show that the effect of fluoride therapy in both groups resulted in significant reductions in the incidence of dental caries. The group receiving two applications of stannous fluoride had a reduction of 100 and 78.2 per cent when evaluated by the DMFT and DMFS indices, respectively. Those children who received the pretreatment with sodium fluoride had a 100 and 60.6 per cent reduction as evaluated by the DMFT and DMFS indices, respectively. These reductions were highly significant, their probabilities being greater than 1 in 10,000. The difference between the two fluoride groups as evaluated by the DMFS index was not significant ($p = 0.07$).

Table III compares the reversals in diagnosis which occurred in the three groups. The children in the control group had a mean number of reversals of 0.420, while the groups receiving two applications of stannous fluoride had a mean number of reversals of 0.839. These values are in keeping with many previous studies which similarly indicated that the use of stannous fluoride is

TABLE III

THE INCIDENCE OF DECAY IN TEETH ERUPTING DURING THE STUDY AND THE REVERSALS IN DIAGNOSIS FOUND IN THE THREE EXPERIMENTAL GROUPS

GROUP	MEAN NO. REVERSALS	CARIES INCIDENCE IN TEETH ERUPTING DURING STUDY PERIOD		
		TOTAL NO. TEETH ERUPTING	TOTAL NO. CARIOUS TEETH	SURFACES
$NaF + SnF_2$	1.032	408	28 (6.9)*	28 (6.9)
SnF_2	0.839	442	22 (5.0)	22 (5.0)
Control	0.420	340	23 (6.8)	23 (6.8)

*Numbers in parentheses refer to the percentages of the teeth or surfaces erupting during the test which became carious.

associated with a greater number of reversals than in similar subjects not receiving stannous fluoride. Our opinion is that such a phenomenon is related to caries arrestment. The group which received the pretreatment with sodium fluoride had a greater number of reversals than the group which received the two applications of stannous fluoride. The reason for this is not known, but is probably associated with the pretreatment effect of the sodium fluoride.

It is interesting to note that the teeth that erupted during the study and which did not receive any topical fluoride therapy similarly did not have any caries reduction. This would suggest that the major anticariogenic effect of the topical fluorides is one which involves some mechanism of combination with the teeth and not subsequent changes in the oral microflora.

The reason why this clinical study did not confirm the in vitro rat study is of considerable interest, and additional tests need to be conducted in order to learn more about such discrepancies. One factor which may bear on this question is the duration of the study. The in vitro data suggested that the pretreatment effect was most pronounced in proving protection for longer periods of time than the single stannous fluoride technic. If it were possible to continue this study for 2 or 3 years, these differences might become apparent.

SUMMARY

Approximately 600 children, aged 6 to 15 years, from Spencer, Indiana, were divided into three groups in order to test the anticariogenic effectiveness of the pretreatment of teeth with sodium fluoride prior to the topical application of stannous fluoride. Group I received one application of a 4 per cent solution of sodium fluoride, followed by a single application of an 8 per cent solution of stannous fluoride. Group II received two applications of stannous fluoride, and Group III was a control. After 1 year there was a significant reduction in dental caries in both groups receiving fluorides. The groups receiving the pretreatment with sodium fluoride had a reduction of 100 and 60.6 per cent, as evaluated by DMFT and DMFS indices, respectively. The group receiving 2 applications of stannous fluoride had a 100 and 78.2 per cent reduction using the same indices. The reasons why these data did not confirm in vitro data indicating the superiority of the pretreatment with sodium fluoride are not known.

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REDUCED ORAL TEMPERATURES AND ACID PRODUCTION RATES IN DENTAL PLAQUES

WILLIAM R. STANMEYER AND ROBERT J. ADAMS

U. S. Naval Medical Research Laboratory, U. S. Naval Submarine Base, New London, Conn.

WHEN Miller[1] presented the chemical-parasitic theory of caries formation, he pointed out that three factors governed the inception and progress of dental decay: (a) microorganisms, (b) the formation and attack by acids, and (c) the resistance offered to the process by forces resident within the tooth.[2] Since the presentation of this theory, many research workers have studied the problem extensively.

With the exception of Gottlieb's[3] proteolytic theory, the generally accepted concept of the inception of dental decay is that accumulations of debris, mucin, and bacteria (dental plaques) occur on protected areas of teeth which, under certain conditions, produce a highly concentrated acid capable of causing enamel decalcification. The rapidity and concentration of acid formation have been attributed to (a) types and numbers of bacteria, (b) rate of salivary flow, (c) buffering capacity of plaque, (d) thickness and composition of plaque, (e) position of plaque, and (f) the relation of food, not only through its chemical action on the teeth but its effect on bacterial activity.[4-9]

Bacteria need not only specific foods for their growth, but their activity depends to a great extent on temperatures. For example, *Lactobacillus acidophilus* produces optimum growth at 98.6° F. and no growth at 68° to 71.6° F. In like manner, *Streptococcus salivarius* and *lactis* exhibit optimum growth at 98.6° F. and very little growth at 41° to 50° F.[10]

During the International Geophysical Year, a dental office was maintained at Little America, Antarctica, as part of the support force to that expedition. Five volunteers were chosen who exhibited the presence of dental plaques on a number of teeth. Preselection of test subjects, as practiced by Ludwig and Bibby,[11] was not possible.

METHOD

The experiment was conducted in July and August, 1957. The men had then been at Little America since January, and had lived and worked at temperatures averaging −14.7° F., −27° F., −23.4° F., −11.1° F., −32° F., and −30.1° F. for the months of March through August, respectively.

The following procedures were carefully followed: (1) A minimum of 120 minutes after eating and 60 minutes prior to going into the cold, approximately

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3 c.c. of unstimulated saliva was collected from each subject. Dilutions were made, and 1 c.c. was plated out using Rogosa's medium[12] for lactobacillus counts. Dental pH values were then measured on the buccal surfaces of the maxillary and mandibular posterior teeth and the labial surfaces of the upper and lower anterior teeth. These measurements were taken at room temperature using a Beckman potentiometer with a dental needle glass electrode. (2) The subjects were then given 75 c.c. of a 20 per cent glucose rinse, which they held in their mouths without agitation for 1 minute, and 15 minutes later the plaque pH values were again taken at room temperature (68° F.). The volunteer then went out of doors for at least 75 minutes in temperatures varying from –31° F. to –57° F. A second 3 c.c. sample of unstimulated saliva was collected for lactobacillus counts while still at cold temperatures. The subject was then given the same quantity of 20 per cent glucose to hold in his mouth for 1 minute. He remained out of doors for another 15 minutes, after which time he was returned to room temperature. Two minutes later the pH values of plaques previously recorded were taken. All instruments were carefully calibrated against known standards before each reading. (3) Tooth temperatures both indoors and out of doors were measured later by means of a direct reading thermocouple unit.

RESULTS

The mean plaque pH of the 176 plaques measured at room temperature was 6.44 with a standard deviation of 0.58. The initial drop in pH following a carbohydrate rinse at room temperature amounted to 0.42, with a mean plaque pH recording of 6.02 and a standard deviation of 0.54. These points are shown as *A-B* on Fig. 1.

At the end of 75 minutes in the cold, the plaque pH values were retaken. The mean found was 6.51 with a standard deviation of 0.33. The second carbohydrate rinse was now given. The subjects remained out of doors for 15 minutes, after which the pH of the plaques was remeasured. The mean remained unchanged at 6.51 with a standard deviation of 0.43. These last two points are shown as *C-D* on Fig. 1. The differences between *A* and *B*, *B* and *C*, and *B* and *D* are, in each comparison, large enough to be quite significant on statistical measures.

A marked reduction in lactobacillus counts was found following the 75-minute period of outside exposure in low temperatures. Results were tabulated for our 5 regular subjects and for 6 others. One man showed zero count both before and after. Counts from 4 men which ranged from 600 to 7,800 at room temperature dropped to zero count following exposure. Six men after exposure showed drops from 5 to 70 per cent of the room temperature counts.

Table I shows the effect of a –5° F. air temperature and mouth breathing on tooth surface temperatures. There was a consistent decline on all teeth during the first 40 minutes of exposure. Upon return to a warm room (+68° F.),

the tooth surface temperatures rapidly rose, approaching the pre-exposure level within 2 minutes. In no instance, was pain reported with these tooth surface temperature variations.[14]

TABLE I.

TOOTH SURFACE TEMPERATURE TAKEN AT ROOM TEMPERATURE (68° F.) AND AFTER EXPOSURE TO −5° F. AIR TEMPERATURE

	TEMPERATURES – DEGREES F.			
	MAXILLARY ANTERIOR TEETH	MANDIBULAR ANTERIOR TEETH	MAXILLARY POSTERIOR TEETH	MANDIBULAR POSTERIOR TEETH
Before exposure	86 (30° C.)	91 (32.8° C.)	86 (30° C.)	90 (32.2° C.)
10 min. exposure	63	72	66	74
20 min. exposure	58	68	58	69
30 min. exposure	60	54	58	59
40 min. exposure	40	47	56	58
50 min. exposure	38	44	56	58
60 min. exposure	35 (1.7° C.)	42 (5.6° C.)	56 (13.3° C.)	59 (15° C.)
Room temp. 1 min.	72	71	74	78
Room temp. 2 min.	76	75	78	80

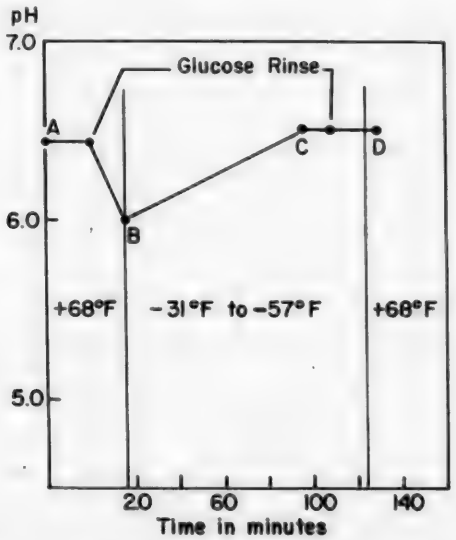

Fig. 1.—Mean pH changes in 176 plaques in 5 subjects following glucose rinse at room temperature and while at temperatures that varied between −31° F. and −57° F. *A*, Pre-rinse pH. *B*, 15 minutes post-rinse pH. *C*, Pre-rinse pH. *D*, pH, 15 minutes after second rinse, plus 2 minutes at room temperature.

DISCUSSION AND CONCLUSIONS

Among the several factors influencing the growth, multiplication, and activity of bacteria is temperature. It cannot be presumed that optimum temperature conditions for bacterial activity exist in all parts of the mouth. The only area in the oral cavity showing near constancy of temperature is under the tongue with the lips closed and at room temperature. The growth of *Lactobacillus acidophilus* is optimum at 37° C. (98.6° F., the temperature under the

tongue with lips closed) and ceases at 20° to 22° C. (68° to 71° F.). The activity of this bacterium in terms of growth and acid production at temperatures between these two extremes is unknown.

Wind conditions were not unusual during the experimental periods, but the average relative humidity was 5 per cent. This low humidity dried the nasal mucous membranes and made breathing difficult. Consequently, as the men remained in these cold temperatures, mouth-breathing became not only the usual practice but also a necessity.

Tooth surface temperatures, while under these conditions, were reduced from near optimum levels for *Lactobacillus acidophilus* to levels considerably below those at which activity or growth will occur. Generally, the posterior teeth registered warmer than the anterior teeth, and the mandibular warmer than the maxillary teeth. It will be noted that the mandibular posterior teeth which were the *least* chilled recorded a temperature of 58° F. after 60 minutes of exposure to cold. This is 10° F. below that at which *Lactobacillus acidophilus* activity is said to occur. This must explain why, following the second glucose rinse, bacterial activity in the form of acid production did not occur even though the tooth surfaces had been allowed to warm to pre-exposure levels before plaque acidity was measured.

Bacterial counts dropped in every instance. Since saliva was collected without stimulation and rinses were given without agitation, lowering of bacterial counts may be accounted for by the fact that as the men breathed cold air, bacterial growth was slowed and bacteria that were swallowed with the saliva[15] were not replaced. Counts retaken the following day, after 18 hours spent at room temperature, approached the pre-cold levels.

Conclusions to be drawn from this study suggest that cariogenic activity as measured by bacterial growth and acid production capacity may be directly related to environmental temperatures. Lowered environmental temperatures are usually associated with lowered humidity resulting in mouth breathing and lowered intra-oral temperatures. These decreased temperatures on tooth surfaces attenuate the activity of the oral acid producing bacteria, thus reducing their potential to initiate the process of caries.

If there is a parallel between our studies and those already completed on the cariogenic rates of the more primitive peoples living in cold climates, their low caries incidence may be further explained. The fact that cariogenic rates increased after contact with civilization may be attributed not only to the introduction of refined food to their diets, but also to the fact that these people have been given tighter dwellings and newer methods of heating them.

SUMMARY

Changes were measured in the pH of 176 dental plaques on men living in Antarctica following a carbohydrate rinse given at room temperature and a second rinse given 75 minutes later. The intervening time was spent in temperatures ranging from –31° F. to –57° F. The first rinse showed the usual increase in plaque acidity. The second rinse produced no such increase, and

thus indicated that the exposure to cold greatly inhibited or completely attenuated bacterial action. Lactobacillus counts taken at room temperature and retaken after the subjects' exposure to the cold showed a marked decrease. Decrease in bacterial action was found to be positively correlated with decrease in tooth surface temperatures.

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SOLUTION RATE OF TOOTH ENAMEL IN AN ACETATE BUFFER

R. S. MANLY AND DOROTHY P. HARRINGTON

Westwood Research Laboratory, Westwood, Mass.

TESTS for enamel solubility in acid have usually been performed by determining the quantity of enamel dissolved within a certain period of time. The early method of Volker[1] employed 0.2 M acetate buffer at pH 4 to produce a weight loss of powdered enamel which was measured after 1 hour. Since that time, other workers have employed the principles of this technic with modifications involving changes in kind, molarity, or pH of the buffer, in the time of contact with enamel powder, or in the analysis of the solution for calcium or phosphorus rather than measurement of weight loss of enamel. In most instances, the investigators have implied that the values obtained represent the solubility of enamel in acid or the rate of solution of enamel in acid. According to strict chemical definition, the term solubility cannot be applied since a steady state is not reached. Rate of solution is a more applicable term, but one that could theoretically vary from the beginning of the experiment to its end. Most testing procedures do not measure such change, for they record only the average effect over the period of time involved.

This investigation was undertaken in order to learn whether there would be value in calculating instantaneous rates of solution from periodic measurements of the concentrations of calcium and phosphorus in the solution. These calculations might be helpful in discovering what factors cause the changes in instantaneous rates of solution during the first 30 minutes of attack. For example, it might be imagined that enamel powder preferentially loses finer and more soluble particles on being attacked by acid and thus develops a greater resistance to decalcification.[2] Alternatively, perhaps the important factor is the increasing concentration of calcium and phosphorus in the buffer. A procedure which would permit a serial determination of calcium and phosphorus concentrations in solution would allow this problem to be studied.

METHODS

Gravimetric Procedure.—Extracted teeth were kept refrigerated, pulverized in a Wiley mill to pass 100 mesh, separated by a centrifugal flotation method,[3] and the enamel was checked for purity. Fifty milligrams of such enamel, 100 to 200 mesh in fineness, and approximately 99 per cent pure, was placed in a tared Selas, porous-bottomed crucible. Ten milliliters of 0.2 M acetate buffer, having a pH of 4.2, was pipetted into the crucible while it rested upon a suction

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flask. A motor-driven glass stirrer was rotated at a sufficient speed to suspend the enamel particles. During the stirring, positive pressure of air prevented the buffer from filtering through the bottom of the crucible. At appropriate periods of time, the acid was drawn off and the enamel powder washed for 2 minutes with distilled water. The crucibles were dried for 2 hours at 100° C., cooled in a desiccator, and reweighed. Etch times were 1, 3, 5, 10, 15, 20, 25, and 30 minutes, and from one to three tests were performed for each etch time. Additional tests were performed on enamel which had been treated with 0.5 per cent sodium fluoride for 3 minutes (corresponding to 0.227 per cent fluoride). Excess fluoride solution was drawn off and the enamel washed for 2 minutes prior to treatment with buffer.

Volumetric Procedures.—Fifty milligrams of enamel was added to a Selas crucible and 10 ml. of the acetate buffer introduced. The solubility test was started by turning on the stirrer. After 1 minute of stirring the stirrer was stopped, one drop of the solution was removed, placed on a glass slide, and filtered through a small piece of filter paper into a medicine dropper. Twenty microliters of the filtered solution was pipetted for calcium determination and 40 μl pipetted for phosphate determination. Calcium was determined by the method of Natelson and Penniall.[4] Phosphate was determined by the method of Fiske and Subbarow,[5] modified to eliminate steps required for removal of protein. Standards for calcium were prepared by dissolving monocalcium phosphate in buffer and those for phosphate by using potassium phosphate in the buffer. Calcium quantities in the sample amounted to 1 to 7 μg and the phosphate was usually determined at 1 to 12 μg. Samples were taken at 1, 3, 5, 10, 15, 20, 25, and 30 minutes. Triplicate runs were performed on untreated enamel and quadruplicate determinations on enamel treated for 3 minutes with 0.5 per cent sodium fluoride.

RESULTS AND DISCUSSION

Loss of Solvent Action by Buffer.—The results of the gravimetric studies are shown in Fig. 1. The middle curve shows the milligrams of enamel dissolved after varying periods. About 20 per cent of the enamel was dissolved in 30 minutes. Treated enamel lost slightly more than 7 mg. during the same period, corresponding to a 30 per cent reduction in amount dissolved. The uppermost curve shows the calculated solubility reduction for periods from 1 to 30 minutes. These reductions were obtained from the ratios of weight losses that were read from smooth curves for treated and untreated enamel. Initially the reduction was only 19 per cent but it rose gradually to a plateau of 30 per cent at 10 minutes, and stayed constant over the period of 10 to 30 minutes. Since most investigators have used contact times ranging from 15 to 30 minutes, this finding is fortunate. A higher value for solubility reduction was obtained by one of the authors in 1949,[6] probably because at that time the fluoride solution was not washed from the enamel prior to the buffer treatment.

The solution rate changed quickly during the first 10 minutes of buffer treatment, either because the enamel became less soluble or because the buffer

lost its power of attacking enamel. Perhaps fine particles of enamel were dissolved preferentially during the first 5 minutes, because of their higher specific surface. This question was answered by checking the constancy of solvent action of the buffer on fresh enamel and the acid resistance of etched enamel with fresh buffer. Samples of enamel were tested in the usual manner using etch times of 10, 20, and 30 minutes. The results are shown in the first line of Table I. Values are percentages dissolved at 10, 20, and 30 minutes. The

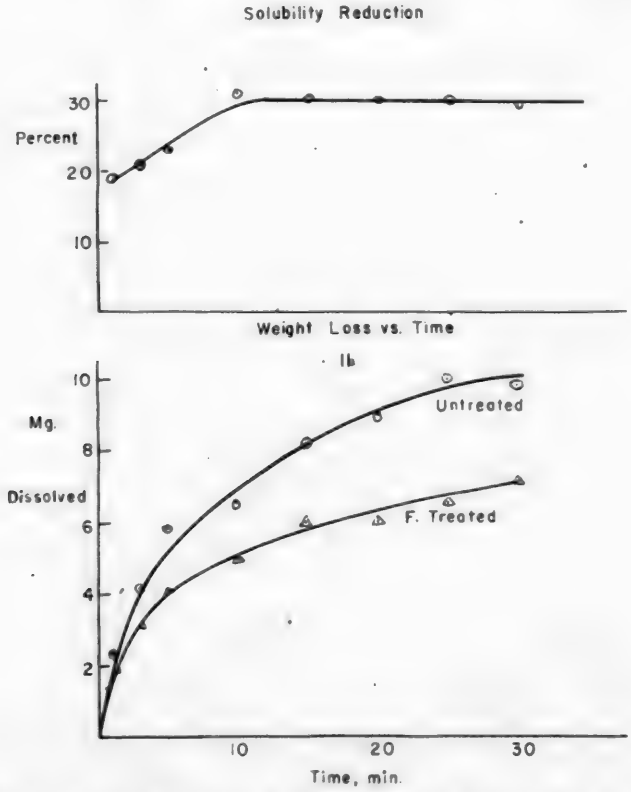

Fig. 1.—Percentage of enamel dissolved by pH 4.2 acetate buffer for the period 1 to 30 minutes. Middle curve shows untreated enamel and lower curve presents data on fluoride-treated enamel. Upper curve of per cent solubility reduction begins at 19 per cent and rises to a plateau for 10 to 30 minutes.

percentage dissolved amounted to 13, 18, and 20 for the three time periods, corresponding to rates of solution of 1.3, 0.9, and 0.7 per cent per minute. In the next test, a 30-minute period was employed but the buffer was recycled by being drawn off at the end of 10 minutes, replaced on the same enamel, restirred for another 10-minute period, drawn off once again, and replaced for a third time. The same percentage solution of enamel occurred, showing that the manipulation involved in recycling did not produce appreciable loss. Next, recycling was repeated with an aliquot of buffer, but a fresh sample

of enamel was employed for each cycle; the amount dissolved by partially spent buffer was not changed by using fresh enamel, hence some property of the buffer was affected. This conclusion was supported through experiments with new buffer and the same enamel sample for three consecutive periods. The 20- and 30-minute etch time produced two and three times as much solution as did the 10-minute etch period. Readings on pH were made on four samples of partially spent buffer after the buffer had been in contact with enamel for 25 to 30 minutes. Three samples were applied to treated enamel and one to untreated enamel. Each sample showed an increase in pH of just 0.1 pH units. Apparently the buffer is changed in solvent action rather than the enamel being increased in acid resistance. The solution of fine particles is not the reason for decrease in solution rate with time.

Ion Concentration in Buffer.—Next calcium and phosphate concentrations in the buffer were determined at different periods of time. Eight determinations of calcium and phosphate were carried out serially on each solution of buffer. The findings in millimoles are plotted in Fig. 2 on the ordinate with time of reaction in minutes on the abscissa. The mean values for solution of calcium and phosphorus from untreated enamel generally fell on a smooth curve. Comparisons were made between our findings and those of Blackwell and Fosdick[2] who used 1.0 M lactate buffer. If we allow for the lesser concentration of buffer used by us, the correspondence between the data is excellent. Between 5 and 30 minutes, the solution rate for calcium in 1 M lactate was 1.76 to 1.93 times as great as our findings with 0.2 M acetate. Comparisons could not be made with the findings of Hills and Sullivan,[7] since the shortest time in their investigation was 1 hour.

TABLE I. PERCENTAGE OF ENAMEL DISSOLVED BY FRESH AND PARTIALLY SPENT BUFFER

BUFFER RECYCLED	FRESH BUFFER EACH PERIOD	FRESH ENAMEL EACH PERIOD	TIME OF ETCH (MIN.)		
			10	20	30
No	No	No	13	18	20
Yes	No	No	—	—	20
Yes	No	Yes	13	18	21
No	Yes	No	13	28	40

Initially there was a high calcium to phosphorus ratio on the molar basis for the solutions we studied; at first it was 1.96, perhaps due to solution of $CaCO_3$. At the third minute, the ratio fell to 1.58 and stayed at this level plus or minus 0.1, with the final ratio of calcium and phosphorus in solution equivalent to 1.62. The theoretical ratio for hydroxylapatite would be 1.66.

The curves for fluoride-treated enamel in Fig. 2 indicate that treated enamel was less soluble during the etch periods from 10 to 30 minutes. This was expected. The behavior during the first 5 minutes was not expected. Here there was little difference between treated and untreated enamel with respect to calcium release, even though the phosphate release was depressed by fluoride. Apparently fluoride treatment affects the release of calcium differently than the release of phosphate. The magnitude of this difference can be seen in Fig. 3. Here the per cent of solubility of treated enamel is plotted against time,

according to the usual method for determining the effect of fluoride on rate of solution of enamel in acid. The smooth curves are calculated from those given in Fig. 2. If fluoride effect were judged from the phosphate determination, the solubility of treated enamel would initially be 70 per cent of the control and would fall within the first 5 minutes to approximately 60 per cent of the control and remain at this level for the 30-minute duration. The behavior of calcium appeared considerably different. During the 3- to 5-minute period

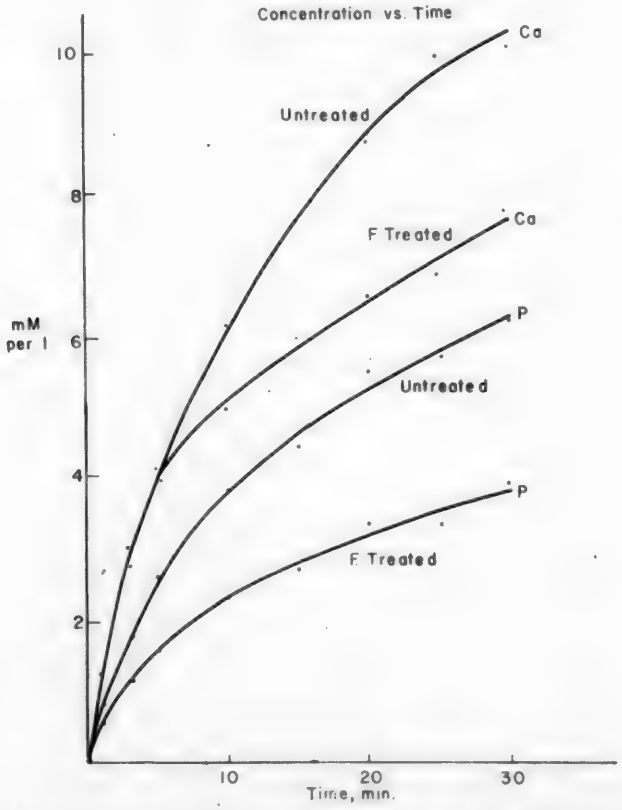

Fig. 2.—Concentration of calcium and phosphate in acetate buffer in contact with powdered enamel for periods of 1 to 30 minutes. Fluoride-treated enamel releases calcium at same rate as control during first 5 minutes, then at a lower rate. Phosphate is less readily released at all times.

there was sometimes more calcium in the etch liquid from the treated enamel than from the control. Between 10 and 30 minutes, the calcium concentration leveled out, showing a 20 to 25 per cent reduction in solubility. There can be large differences in the magnitude of solubility depression by fluoride treatment, depending upon whether calcium or phosphate is used as the criterion and, in case calcium is used, whether the values are based on a 3- to 5-minute etch time, or some other time.

Solution Rate of Calcium and Phosphate in Buffer.—These findings seem to justify calculation of instantaneous rates of solution. This calculation would eliminate some types of misinterpretation from the data. The usual calculations of percentage reduction of solubility involve a summation of all rates of solution from zero time to the time at which the reaction is stopped. The concentration of ions in solution at any moment is determined by the rates of release of ions during the whole preceding period. The rapid solution of calcium during the first 5-minute period adds a base line of calcium which raises all values obtained in later periods. Calculation of instantaneous solution rates would eliminate this possibility of misinterpretation.

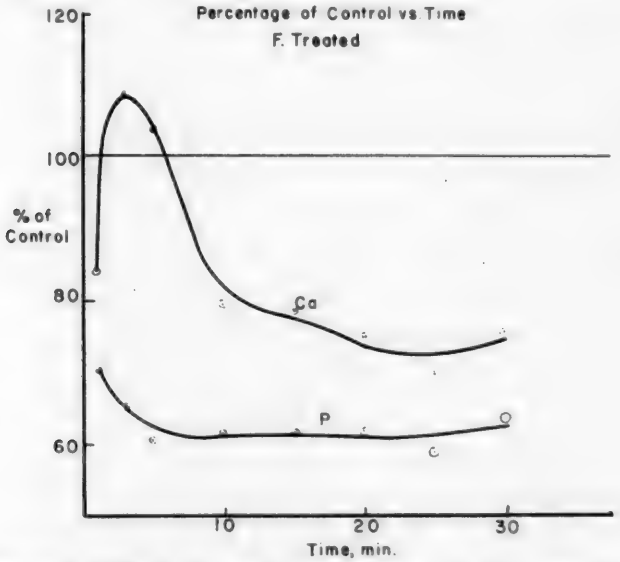

Fig. 3.—Percentage solubility of treated enamel vs. time of etch. Calcium and phosphate in solution are depressed except for calcium during first 5 minutes.

Instantaneous rates of solution were obtained graphically by placing a straight edge against the smooth curves in Fig. 2 and reading off the slopes of the line at the eight different time periods. Then the rate for treated enamel was divided by the rate for control enamel and multiplied by one hundred to provide the relative rate of solution $\left(\frac{R_T}{R_U} \times 100\right)$. The original rates were calculated as increases in millimoles of calcium per minute. The solid line of Fig. 4 shows fluoride-treated enamel. During the first 3 minutes there was no change in solution rate. Between 5 and 20 minutes the solution rate was depressed, and began to rise toward the original solubility rate after that period Values became uncertain at 25 and 30 minutes because of errors in obtaining slopes that approached zero. Fluoride reduced the instantaneous solubility rate of calcium by 50 per cent during the 10- to 20-minute period. Comparison

of the calcium concentrations, on the other hand, gives the impression that over-all solubility was reduced by 20 to 30 per cent. Apparently the instantaneous rate varies widely with time and it is only during a rather brief period that the rate is depressed.

Influence of Fluoride on Solution Rates.—According to McCann and Bullock,[8] the treatment of tooth enamel with 0.5 per cent sodium fluoride solution should cause deposition of approximately 0.4 per cent fluoride on enamel, mostly present as calcium fluoride. Perhaps the first event to take place when fluoride-treated enamel is placed in buffer, is the dissolution of some calcium fluoride.

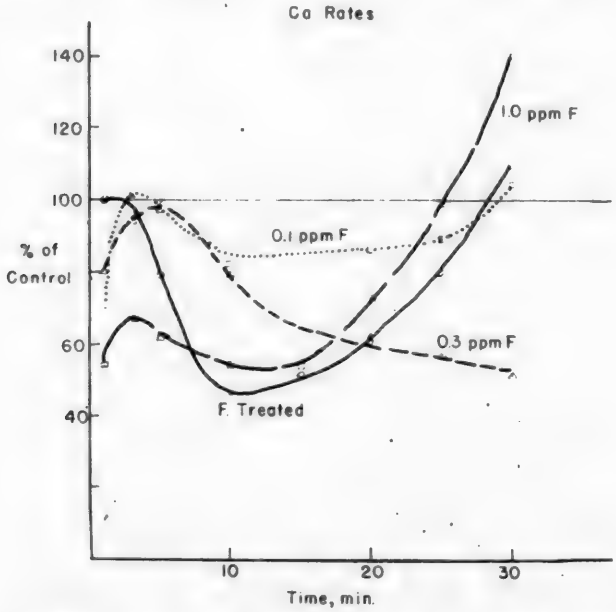

Fig. 4.—Relative instantaneous rates of solution for calcium. Rates vary widely for fluoride-treated enamel, and appear similar to rates obtained with buffer containing 0.3 to 1.0 ppm of fluoride.

This would account for the high initial solubility rate of calcium during the first 3 minutes. Some of the dissolved fluoride might remain in solution and some might be absorbed again on etched enamel. The mode of action of fluoride on solubility rate will depend on the proportion entering the solution and remaining there. One approach to the question is via the addition of fluoride to the buffer initially. Perhaps small quantities of fluoride in solution would act more slowly than fluoride combined with treated enamel, suggesting that the fluoride must be absorbed in order to exert its action. Results on studies of this type are shown on the remaining three curves on Fig. 4; these curves were derived from experiments in which untreated enamel was reacted with buffer to which fluoride had been added. It appears that as little as one tenth of a part per million of fluoride may have had some transient effect on the rate of

calcium solution. Three tenths of one part per million was obviously influential in depressing solution of calcium, and one part per million of fluoride may actually have had an effect which was greater than that of fluoride-treated enamel; depression of solubility occurred immediately and did not require 5 minutes for the fluoride effect to be observed. This finding does not support the need for adsorption prior to solubility-depressing action.

Fluoride treatment may improve resistance of enamel to attack, not because of inherent lesser solubility of the solid, but because the solid gives up calcium fluoride to the buffer, and this added fluoride either inhibits the solvent power of the buffer or is redeposited at areas where decalcification is taking place.

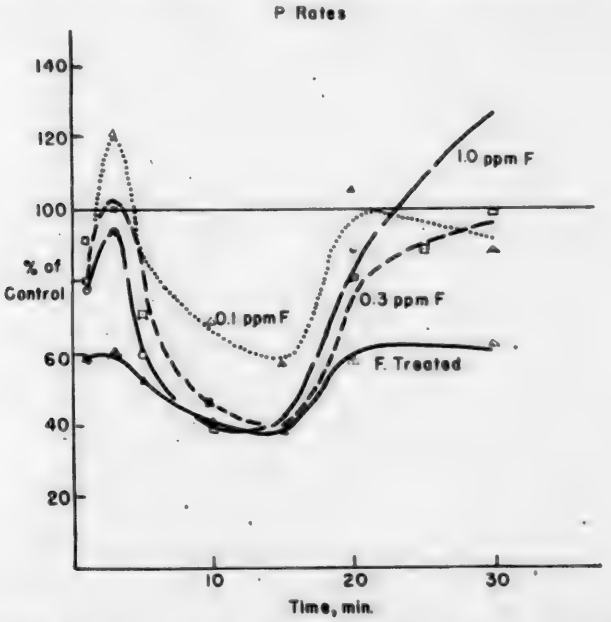

Fig. 5.—Relative instantaneous rates of solution for phosphate. Fluoride-treated enamel is uniformly low, and is matched by fluoride in solution only during period of 5 to 20 minutes.

The solution containing 0.3 part per million of fluoride contained 3 μg of this element. If the total were completely adsorbed on 50 mg. of enamel there would be only 0.006 per cent fluoride on the enamel, much less than the 0.4 per cent which was presumably placed there by the fluoride treatment. At first it appeared easier to explain fluoride's action through its effect in solution rather than from its effect on enamel. Yet the recent paper by Brudevold, Hein, Bonner, Nevin, Bibby, and Hodge[9] reported that fluoride was very rapidly adsorbed, with an average of 28 per cent of the fluoride in solution adsorbing on an enamel surface within 1 minute; adsorption appeared to be more rapid both from an acid solution, and on interior enamel with a roughened surface. The conservative interpretation of our findings is, therefore, according to the following mechanism: CaF_2 of treated enamel dissolves promptly in buffer, but

the fluoride ion is very quickly and preferentially adsorbed on etched, roughened surfaces on the enamel. This relocation of fluoride places it at sites on the powder where decalcification is most rapid. Its deposition here causes a major depression of over-all solubility. When fluoride is initially in solution, then deposition at etch sites can take place immediately and need not wait for CaF_2 to dissolve.

Solution rates were also calculated for phosphate from the slopes of phosphate curves in Fig. 2 and expressed as per cent of control rates in Fig. 5. Fluoride treatment caused a depression of phosphate solution rates within 1 minute, perhaps because phosphate had already been removed from the surface by fluoride exchange when calcium fluoride was formed. Solution rates of treated enamel were depressed about 40 per cent except for the period from 10 to 15 minutes, when the rate was depressed 60 per cent. The addition of 0.3 and 1.0 parts per million of fluoride to the solution produced similar depression in rates of solution of phosphate during the period of 10 to 20 minutes but, beyond these limits, fluoride in solution was less effective than fluoride on treated enamel.

These experiments did not provide any definitive information as to whether one part per million of fluoride in buffer exerts its action on the solvent power of the buffer or by a very prompt adsorption at sites of active decalcification. The results do suggest a manner in which fluoride in drinking water might be able to reduce dental caries. If water containing one part per million of fluoride were consumed at a time when the acid decalcification of enamel by plaques was taking place, diffusion of this concentration of fluoride in the plaque would be expected to depress the rate of enamel decalcification and perhaps affect the rate of initiation of new lesions.

SUMMARY

Rate studies on enamel solubility in pH 4, 0.2 M, acetate buffer have been carried out by a gravimetric method and by colorimetric determination for phosphate and calcium in the solution. There was a gradual loss of weight of powdered enamel in a nonlinear fashion from the first to the thirtieth minute. Solubility reduction by fluoride treatment ranged from 19 per cent during the first minute to 30 per cent for the period from 10 to 30 minutes.

Tests with fresh and partially reacted enamel and buffers indicated that the gradual slowing in solution rate is attributable to a change in solvent action of the buffer with time rather than a change in the acid resistance of the enamel.

Procedures were developed for microdetermination of calcium and phosphate serially on small portions of a buffer solution at eight times ranging from 1 to 30 minutes after a test was started. There was a gradual decrease in the solution rate of calcium and phosphate for untreated enamel. Treated enamel had lower solution rates for phosphate throughout the time period and for calcium from the tenth to the thirtieth minute. Treatment did not appear to affect calcium solution rate during the period from 1 to 5 minutes. Findings indicated

that solubility reduction by fluoride depends upon the duration of the experimental period and whether the effect is judged from determination of weight loss, or calcium or phosphate concentration in buffer.

Instantaneous solution rates for calcium and phosphate in buffer were calculated by drawing tangents to the curves for concentration vs. time. Wide variation in solution rates occurred during the period of 1 to 25 minutes. Tests with 0.1, 0.3, and 1.0 parts per million of fluoride in the buffer indicated considerable influence from traces of fluoride and suggested that fluoride reacts preferentially at high dilutions with sites in tooth enamel that are undergoing decalcification.

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METHODS OF INVESTIGATING THE VASCULAR ARCHITECTURE OF THE MANDIBLE

LAWRENCE COHEN

Institute of Clinical Research, Middlesex Hospital, London, England

A KNOWLEDGE of the blood supply of a bone is important in the understanding of pathologic processes which involve it. A detailed study of the literature reveals a lack of knowledge concerning the vascular architecture of the mandible. It was for this reason that the present investigation was undertaken.

MATERIALS AND METHODS

In this investigation most of the experimental work was carried out on adult cats. Adult animals were chosen in preference to kittens because the presence of deciduous teeth would have added difficulties to the interpretation of the findings. Fresh material was used and the animals were sacrificed by the injection of intraperitoneal Nembutal. Twenty full-grown cats were used.

To demonstrate the vascular architecture of the mandible of the cat, two methods were used:

1. The injection of one or other of the following materials into the common carotid artery—(a) radiopaque material, (b) carmine gelatin and (c) India ink.
2. The injection of radiopaque material directly into the cancellous tissue of the bone. By this method it was hoped to demonstrate the venous drainage.

To supplement the findings made in the cat, one dog head and one monkey head, from animals already killed for another investigation, were injected on one side only, a limitation imposed by the heart and some of the great vessels having been removed in connection with prior study. The dog was injected with carmine gelatin and the monkey with India ink in gelatin.

It could be argued that any conclusions relating to the vascular architecture of the mandible of the cat, the dog, and the monkey might not apply to the human mandible. For this reason, some human material was used in order to see whether or not the conclusions reached by animal experiment agreed with those in man.

One human mandible was removed from the body postmortem and injected with India ink in gelatin to demonstrate the vascular architecture, and 5 cadavers were injected with radiopaque material directly into the cancellous bone of the mandible to demonstrate the venous drainage.

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1a. *The Injection of Radiopaque Material Into the Common Carotid Artery of the Cat.—*

The radiopaque medium: Barclay[1] found that if a radiopaque medium such as Thorotrast or silver iodide was injected into an artery, the capillaries were filled. In the resulting radiograph the filled capillaries obscured the arteries. Accordingly, to obtain a clear radiograph of the finest arterioles, it is necessary to use a medium which does not enter the capillaries. Schlesinger,[2] in a study of the coronary arteries, used a lead-agar mass which penetrated to vessels with a diameter of 0.04 mm. There are two preparations which penetrate consistently as far as vessels of 0.03 mm. diameter. One is bismuth oxychloride suspension used by Evans[3] and the other is finely divided Micropaque* (barium sulfate) used by Harrison and Asling[4] in their investigations of the blood supply of the adrenal gland.

A 50 per cent suspension of Micropaque fills the smallest arterioles, but is scarcely sufficiently radiopaque. An 80 per cent suspension (with 3 per cent gelatin) gives a satisfactory image and penetrates to 0.03 mm.[5] In the present investigation bismuth oxychloride was used.

Technique:

The Radiopaque Medium: The bismuth oxychloride-gelatin suspension was prepared in three stages. First a suspension of bismuth oxychloride was prepared by interaction of a solution of bismuth carbonate (515 Gm.) in hydrochloric acid (1,050 ml.). This was then filtered into a large volume of water and the bismuth oxychloride was precipitated. The precipitate was washed free from acid by decantation and allowed to settle to a volume of 1,000 ml. The supernatant water was then decanted. Next, a solution was prepared by heating powdered gelatin (750 Gm.) and methyl hydroxybenzoate (3 Gm.) in water (750 ml.). Finally, the bismuth suspension was added to an equal volume of the gelatin solution, avoiding the introduction of air, and this was strained through muslin into final containers. The ultimate suspension contained approximately 25 per cent bismuth oxychloride and 30 per cent gelatin.

The Injection: A midline incision was made in the neck of the recently killed animal and the common carotid artery of each side was exposed. One common carotid was ligated and the other cannulated. The jugular vein of the cannulated side was cut and the blood was then washed out of the head with normal saline at a temperature of 70° C. and a pressure of 200 mm. Hg using compressed air from a cylinder. When the returning fluid ran clear, a bismuth oxychloride gelatin suspension was injected at a temperature of 70° C. and at a pressure of 200 mm. Hg. The aim was to produce complete distention of the arteries and arterioles.

The cat was placed in a refrigerator overnight to allow the gelatin to harden. The mandible was then dissected out and the periosteum and soft tissues scraped off. The object of this procedure was to prevent the periosteal vessels from obscuring the vessels in the bone when the mandible was radiographed. The specimen was then fixed in 5 per cent formalin for 48 hours.

*Damancy & Co., Ware, Herts, England.

Following fixation, the mandible was divided into two through the symphysis. The two halves were then decalcified in 15 per cent formic acid in 5 per cent formalin using radiographic control. The decalcifying solution was changed daily. It is necessary to decalcify the mandible before radiography as otherwise the arterioles cannot be distinguished on the radiographs.

Radiography: The two halves of the mandible were radiographed on envelope-wrapper Kodaline* standard film. With focal-film distance of 60 cm.

Fig. 1.

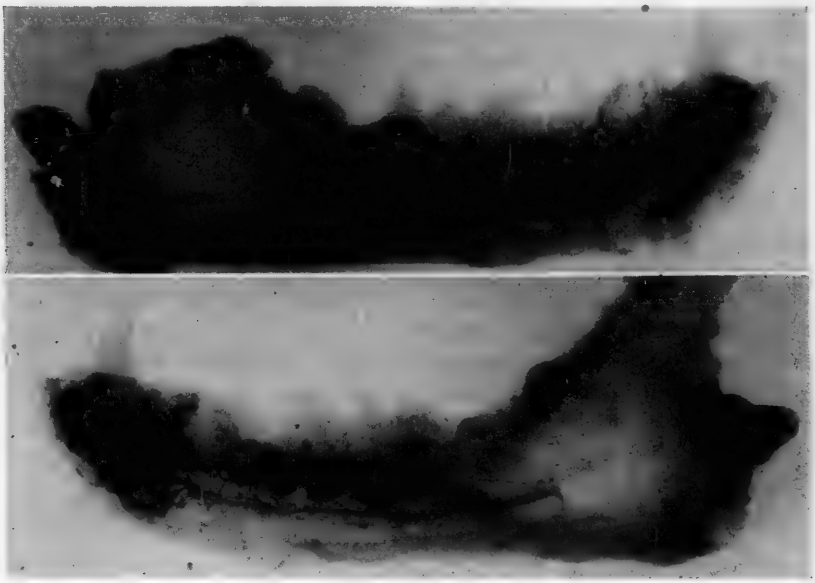

Fig. 2.

Fig. 1.—Positive print of radiograph of right half of the decalcified mandible of a cat injected with warm bismuth oxychloride in gelatin. The coronoid process was fractured during removal of the mandible. (×4; reduced ⅔.)

Fig. 2.—Positive print of radiograph of left half of the decalcified mandible of a cat injected with warm bismuth oxychloride in gelatin. (×4; reduced ⅔.)

(24 inches), the exposure factors were 90 kv., 100 Ma., and 2.5 seconds. Standard x-ray developer gave satisfactory result. Kodaline standard film was used as it is a fine-grain film which allows considerable enlargement of the radiographs to be made.

Photography: The radiograph was enlarged five times and photographed. This showed in finer detail the pattern of the branching arterioles.

1b. *The Injection of Carmine Gelatin Into the Common Carotid Artery of the Cat.*—

The injection of a colored and insoluble substance into blood vessels has been used for many years. The preparation of most injection masses is often empirical while the success of the injection itself is dependent on various factors: of these pressure and temperature of the mass are all important. There

*Kodak Ltd., London.

are two methods of injection—the "cold" and the "warm." The latter is the better and was used in this investigation. The principle of the warm method is the distention of blood vessels with a solution of warm gelatin which solidifies on cooling. To show the path taken by the injection mass, some insoluble dye, usually carmine or prussian blue, is added to the gelatin. For satisfactory injections the mass must be colloidal; if the particles are too large the capillaries will not be injected, while if the mass is in a state of true solution it is liable to diffuse through the vessel walls.

Preparation of the injection mass[6]*:* 1. A little distilled water was added to 150 Gm. of powdered carmine and made into a paste. This was put into a bottle and 75 ml. of distilled water poured on. The mixture was well shaken and concentrated ammonia was added until the carmine had dissolved. Some 10 to 15 ml. was usually needed.

2. Gelatin, 250 Gm., was soaked in 750 ml. of distilled water. Its solution was completed by stirring on a waterbath, keeping the temperature at about 60° C.

3. The carmine solution (also warmed) was added to the warm gelatin solution and mixed thoroughly.

4. Thirty per cent acetic acid was poured in, a milliliter at a time, the mixture being stirred continuously. The mass gradually lost its red transparency and became opaque, and the smell of ammonia diminished. The acid was added until only a faint odor of acetic acid persisted.

5. The hot mass was filtered through several thicknesses of gauze into a 1-liter bottle containing 2 Gm. of phenol crystals.

Technique of injection: The technique was the same as in the injection of bismuth oxychloride. The injection was stopped when the tongue, palate, and gingivae were colored by the suspension and the carmine gelatin was seen to be flowing freely from the cut jugular vein.

The cat was placed in a refrigerator overnight to allow the gelatin to harden. The mandible was then dissected out.

Celloidin sections of one half of the mandible were cut in a coronal plane at 200 μ. The other half of the mandible was sectioned at 100 μ in a sagittal plane. The sections were cleared and mounted.

1c. *The Injection of India Ink in Gelatin Into the Common Carotid Artery of the Cat* (Fig. 3).—

It was found that the injection of undiluted India ink was unsuitable for the demonstration of the vascular architecture of the cat's mandible. During the prolonged histologic techniques of fixation, decalcification, washing, dehydration, and clearing, a large quantity of the India ink was washed out. The explanation of this is that India ink has a particle size of 1 μ. If India ink in gelatin is injected, the India ink is retained in the blood vessels.

Preparation of the injection mass: India ink in gelatin should be freshly prepared as bacterial decomposition of the gelatin takes place on standing. Fifteen grams of gelatin was placed in a flask and to it was added 50 ml. of Kandahar* black India ink and 50 ml. of water. The flask was placed in an oven at 60° C. overnight. In the morning, the hot mass was thoroughly agitated and then filtered through several thicknesses of gauze and placed in a corked bottle until ready for use.

Technique of injection: A similar technique was carried out as in the injection of bismuth oxychloride and carmine gelatin. The injection was stopped when the India ink was observed to be flowing freely from the cut jugular vein.

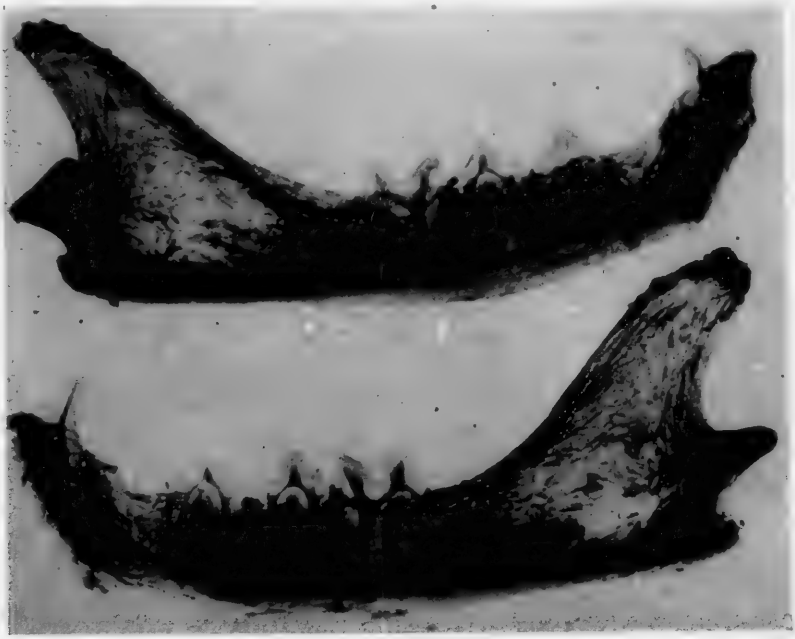

Fig. 3.—Right and left halves of mandible of a cat injected with India ink in gelatin. Specimens are decalcified and cleared in methyl salicylate. (×2; reduced ½.)

The animal was placed in a refrigerator overnight to allow the gelatin to harden and the mandible was dissected out on the following day. The soft tissues and periosteum were scraped off.

Method of preparation: Following the usual histologic technique of fixation, decalcification, and dehydration, the specimen was cleared in xylol. After clearing, the mandible was divided through the symphysis. The two halves were mounted in a glass specimen jar containing methyl salicylate. The latter is used both as a de-alcoholizer and clearing agent and as a solvent of paraffin. It has a refractive index of 1.53 and this fact allows the injected blood vessels

*George Rowney & Co., Ltd., London.

to be visualized through the decalcified bone. The specimen was photographed and the photograph enlarged to twice the size of the original so that the vascular pattern could be identified.

2. *Injection of Micropaque Into Cancellous Bone of the Cat Mandible to Demonstrate the Venous Drainage* (Figs. 4 and 5).—

The technique of Harrison and Gossman[7] was employed. The animal was sacrificed with intraperitoneal Nembutal. A Sahla needle was introduced through the cortical plate of the mandible into the cancellous bone. The Sahla needle was then connected to a 20 ml. Luer syringe containing Micropaque and an injection of 2 ml. was made into the mandible.

The site of the injection and ascending ramus of the injected side were radiographed immediately after the injection.

Fig. 4.

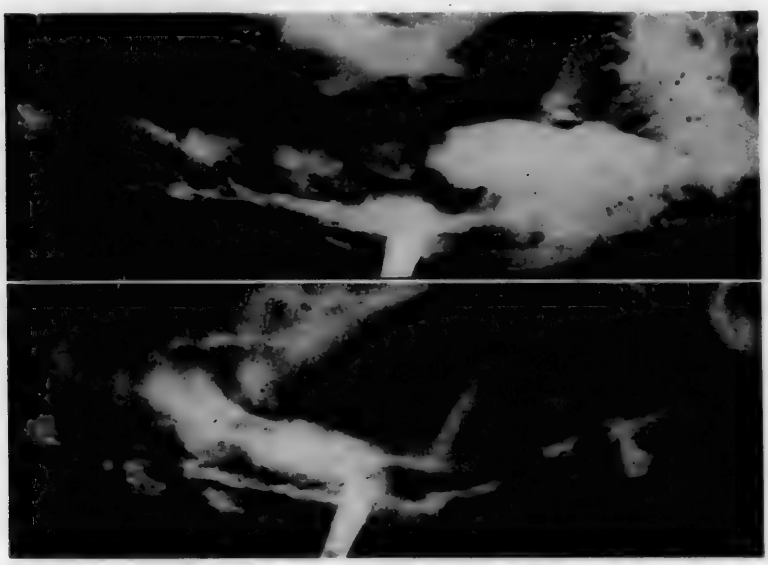

Fig. 5.

Fig. 4.—Radiograph of the right side of a mandible of a cat injected with Micropaque. A single venous channel is shown. (×2; reduced ⅔.)

Fig. 5.—Radiograph of the right side of a mandible of a cat injected with Micropaque. A single venous channel is shown. (×2; reduced ⅔.)

SUPPLEMENTARY STUDY

1. *The Injection of Carmine Gelatin Into the Common Carotid Artery of a Dog* (Fig. 6).—

The dog had been sacrificed for another investigation in which the heart and some of the great vessels had been removed. The right common carotid artery was found and cannulated and the head washed out with normal saline using the same apparatus as in the injection of bismuth oxychloride. Carmine gelatin was then injected.

The animal was placed in a refrigerator overnight to allow the gelatin to harden and the mandible was dissected out on the following day.

Method of preparation: The subsequent treatment was identical to that described for the cat mandible injected with India ink in gelatin.

2. *The Injection of India Ink in Gelatin Into the Common Carotid Artery of a Monkey* (Figs. 7 and 8).—

The thyroid gland of a monkey had been removed for another investigation and the animal then placed in a refrigerator for 24 hours. On removal from the refrigerator, it was placed in warm water for 2 hours before injecting the vessels. It was found that the heart and some great vessels had been removed.

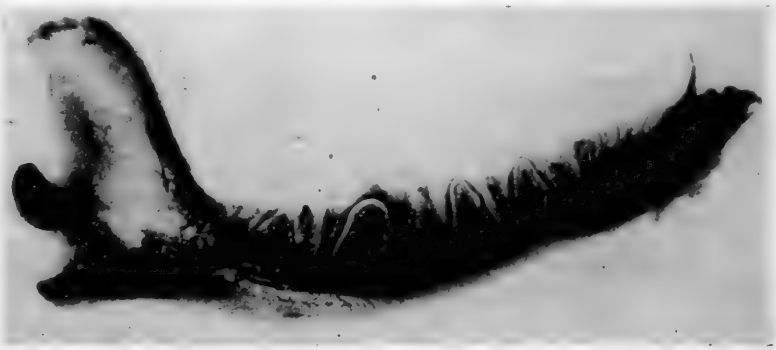

Fig. 6.—Right side of a mandible of a dog injected with carmine gelatin. Specimen is decalcified and cleared in methyl salicylate. (Natural size; reduced ½.)

The left common carotid artery was found and cannulated. After washing out the vessels with normal saline, India ink in gelatin was injected.

The animal was placed in a refrigerator overnight and the head was removed from the body on the following day.

Method of preparation: The subsequent treatment was identical to that described for the cat mandible injected with India ink in gelatin.

3. *The Injection of India Ink Into the Inferior Dental Artery of a Human Mandible Obtained Postmortem* (Figs. 9 and 10).—

A mandible was obtained postmortem from a male aged 81 years whose death was due to coronary ischemia $\overline{4321}\,|\,12345\ \overline{8}$ were present. The left inferior dental artery was found to be missing, this presumably having been removed at postmortem examination. The right inferior dental artery was cannulated. It was found impossible to wash out the blood from the mandible using normal saline with the usual compressed air apparatus but the blockage was finally overcome by forcing normal saline under pressure from a 20 ml. syringe and the vessel washed out. India ink in gelatin at a temperature of 70° C. was then injected using the same syringe. It was noted that India ink

flowed from both mental foramina and also came up the periodontal membrane of all the anterior teeth, i.e., $\overline{4321}\,|\,\overline{1234}$. Some ink flowed from the region of the genial tubercles. The mucosa assumed a bluish hue in the anterior region

Fig. 7.

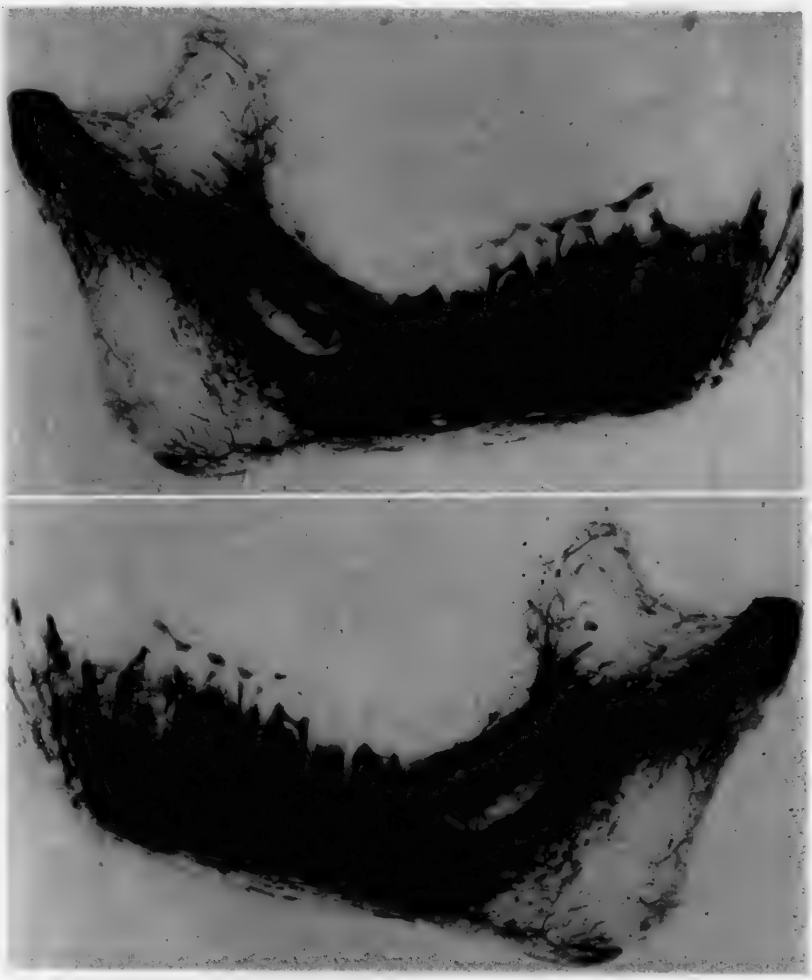

Fig. 8.

Fig. 7.—Buccal view of the right side of a monkey mandible injected with India ink. Specimen is decalcified and cleared in methyl salicylate. (×2; reduced ⅔.)
Fig. 8.—Lingual view of mandible in Fig. 7.

of the mandible but not in the posterior region. The latter is supplied by the buccal branch of the internal maxillary artery.

Method of preparation: The subsequent treatment was identical to that described for the cat mandible injected with India ink in gelatin.

4. *The Injection of Micropaque Into the Cancellous Bone of the Mandible in Cadavers to Demonstrate the Venous Drainage* (Fig. 11).—

To demonstrate the venous drainage of the mandible, Micropaque was injected directly into the cancellous bone of the mandible in cadavers.[8]

Fig. 9.

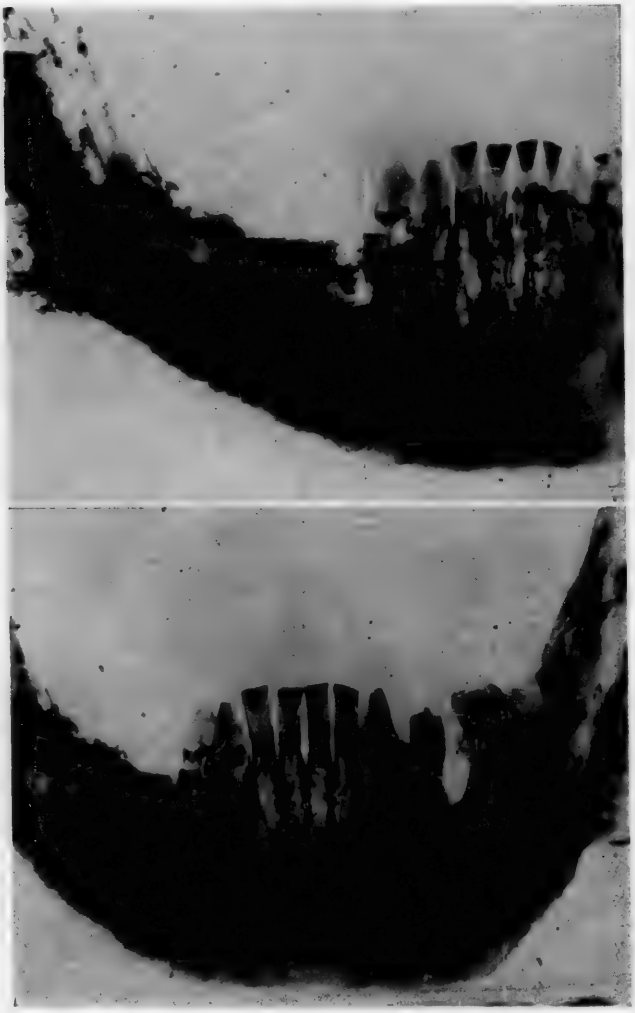

Fig. 10.

Fig. 9.—Labial view of the right side of a human mandible injected with India ink. The specimen is decalcified and cleared in methyl salicylate. (Natural size; reduced ⅓.)
Fig. 10.—Lingual view of above specimen.

DISCUSSION

India ink in gelatin and carmine gelatin have a particle size of approximately 1 μ and, when injected into the vascular system, will traverse the capillaries. These materials can be used to demonstrate both the arterial and venous

systems (Figs. 3, 6, 7, and 8). The bismuth oxychloride suspension used in this investigation has a particle size of 30 μ and thus cannot enter the capillaries most of which have a diameter of 8 to 10 μ. By injecting this material into the common carotid artery of a cat, an arteriogram of the inferior dental artery can be obtained (Figs. 1 and 2). It is necessary to explain how the bismuth oxychloride suspension can be observed to be flowing out of the cut jugular vein when it has not passed through the capillaries of the head and neck. The explanation offered is that the suspension passes into the venous system by traversing arteriovenous anastomoses; these have been demonstrated in the

Fig. 11.—Radiograph of the right side of mandible of a cadaver injected with Micropaque. Note needle in situ. (Half natural size; reduced ¼.)

tongue and inferior turbinate of the nose of other animals[9] and it is reasonable to assume that they are also present in the cat. The presence of arteriovenous anastomoses in the mandible has never been demonstrated and thus it can be accepted that Figs. 1 and 2 are photographs of the arterial system of the mandible.

It was found that the histologic sections of mandibles injected with carmine gelatin were superior in detail to those injected with India ink in gelatin.

If the photographs of a cat's mandible injected with India ink in gelatin (Fig. 3) are examined, the findings may be contrasted with those of bismuth oxychloride-injected specimens (Figs. 1 and 2). The blood vessels shown are only those in the bone, as the overlying soft tissues were removed.

1. The most striking observation is that many more blood vessels are shown. This is due to the fact that India ink traverses the capillaries which bismuth oxychloride does not.

2. The majority of the blood vessels which arise from the inferior dental artery within the mandibular canal pass upward toward the alveolar border. Some vessels which appear to be arising from the inferior dental artery pass toward the lower border of the mandible but these latter vessels are few in number in the bismuth oxychloride-injected specimen and it must be concluded that branches of the inferior dental artery to the lower border of the mandible are small (i.e., less than 30 μ) and of little consequence.

3. Fig. 3 represents the vascular architecture of the mandible. The India ink flows from the inferior dental artery into the capillaries and thence into the mandibular veins. It can be observed in the bismuth oxychloride-injected specimens that the inferior dental artery appears as a thin black line. In the India ink injected specimen, the artery is not so well defined, for numerous vessels surround it in its course through the mandibular canal. These almost certainly represent venae comitantes of the inferior dental artery.

4. The coronoid process, the condyle, and angle of the mandible are heavily stained with India ink. These areas are the sites of insertion of the temporal, lateral pterygoid, and medial pterygoid muscles, respectively, and it may be concluded that the blood vessels which supply these muscles enter the bone at their insertions and supply the mandible. Microscopic examination of sectioned material confirms this.

These vessels must be smaller than 30 μ in diameter as they are not well shown in the bismuth oxychloride-injected specimens.

5. In the ascending ramus, a plexus of blood vessels may be observed. This contrasts markedly with the bismuth oxychloride-injected specimens which in this region are relatively free of injected blood vessels. Thus, it may be concluded that the injected vessels of this region are less than 30 μ in size and are in continuity with the blood vessels of the muscles which are inserted into the ascending ramus. These vessels are not derived from the inferior dental artery.

It will be noted in Figs. 1, 2, 3, 6, 7, 8, 9, and 10 that the majority of the blood vessels arising from the inferior dental vessels pass upward to the alveolar border and that the lower border of the mandible is poorly supplied by blood vessels.

The author believes that the lower border of the mandible is supplied mainly by periosteal vessels. This has been confirmed by histologic examination.

The retrograde injection of bismuth oxychloride suspension into the external jugular vein of a cat was tried as a means of demonstrating the venous system of the mandible. It was hoped to fill the inferior dental veins via the pterygoid plexus. This method was unsuccessful as the injection mass flowed into the veins of the soft tissue in preference to those of the mandible. The injection of Micropaque into the cancellous bone of the mandible revealed the venous system of the mandible. In the human (Fig. 11) but not in the cat (Figs. 4 and 5), it was found that the injected material drained rapidly into the regional superficial and deep veins. The needle in its passage through the

bone must tear the veins and capillaries of the cancellous bone. When the Micropaque is injected into the bone it enters the large capillary network in the bone and is rapidly conveyed via the veins of the periosteum to the veins of the face and neck. In the cat, only a single venous channel in the mandible was demonstrated by this method.

SUMMARY

In this investigation into the vascular architecture of the mandible, the adult cat was used as the experimental animal. To supplement the findings, dog, monkey, and human material were used. The various methods employed are discussed in detail.

It was observed that the majority of the blood vessels arising from the inferior dental vessels pass upward to the alveolar border and that the lower border of the mandible is poorly supplied by blood vessels. The author believes that the lower border of the mandible is supplied mainly by periosteal vessels. The ascending rami of the mandible are supplied by vessels derived from the muscles of mastication which are inserted therein.

I should like to thank the Clinical Research Committee of the Institute of Clinical Research, Middlesex Hospital, for facilities granted in the Institute. My thanks are also due to Mr. W. Warwick James for suggesting the investigation and to Professor E. W. Walls, Mr. J. W. Schofield, and Mr. D. Greer Walker for criticism and advice. I am grateful to Mr. R. A. Kimber for his technical assistance.

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SURFACE REPRODUCTION OF ELASTIC IMPRESSIONS

HIROYASU HOSODA AND TAKAO FUSAYAMA

Tokyo Medical and Dental University, Tokyo, Japan

INTRODUCTION

THE ability to reproduce a smooth surface and accurate detail on stone models is one of the most important factors for the impressions used in indirect inlay or crown technics.

The use of various elastic materials in indirect dental technics is now rapidly spreading. The alginate is the most simple to use, but it has been reported that its surface reproduction is less reliable.[1] The mercaptan rubber-base materials have reportedly produced the best model surfaces.[2] If a fine accurate model surface could be reproduced with an alginate, it would be a great advantage.

In this study, the technical factors which reproduced the smoothest surface and finest detail on the stone model with an alginate impression were investigated; and the results from alginate impressions were compared with those from mercaptan rubber-base impressions.

To test the detail reproduction, Miller and his co-workers[3] used a fine line 15/10,000 of an inch (40 μ) wide cut on an original model surface, and suggested that an impression failing to reproduce this wide a line on a stone model might be inadequate for an indirect dental technic. Modifying his method, lines as fine as 8 to 40 μ were used to compare surface reproduction of detail in this study.

TABLE I

TESTED MATERIALS

MATERIALS	BRAND	BATCH NO.	MANUFACTURER	W/P RATIO
Alginate 1	Technicol	AM19, AL23	G. C. Co. (Japan)	2.5
Alginate 2	Algix		Fujimori Co. (Japan)	2.5
Alginate 3	Inlay-Jeltrate	36555	Caulk Co. (U.S.A.)	2.5
Alginate 4	D.P.I. Cream	2976	D. P. Co. (U.S.A.)	2.1
Alginate 5	Estergin	370619	Takeuchi Co. (Japan)	
Mercaptan 1	Surflex	AG30	G. C. Co. (Japan)	
Mercaptan 2	Coeflex	C4	Coe Lab. (U.S.A.)	
Hydrocal 1	Plastone	AL31	G. C. Co. (Japan)	0.30
Hydrocal 2	Hydrogips	R335	Shofu Co. (Japan)	0.32
Hydrocal 3	Diolite	651RA3933	Kerr Co. (U.S.A.)	0.30
Densite 1	Surstone	AO16	G. C. Co. (Japan)	0.23

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MATERIALS

Five alginate impression materials, two mercaptan rubber-base impression materials, three quick setting stones of hydrocal type, and a hard stone of densite type, made either in the U.S.A. or Japan, were used as listed in Table I.

Alginates 1 through 4 were powder mixed with water, while Alginate 5 was paste mixed with plaster.

PROCEDURE

A brass plate of 3 by 50 by 30 mm. size was used as an original model (Fig. 1, *left*). Very fine lines of varying widths were cut by using a steel cutter having a tip the sides of which met at 90 degrees. Spots, exactly 8, 10, 20, 30, or 40 μ in width on these lines, were determined by measuring with a Ramsden ocular micrometer and the reproduction of these spots on stone models was compared under a microscope (Fig. 1, *right*).

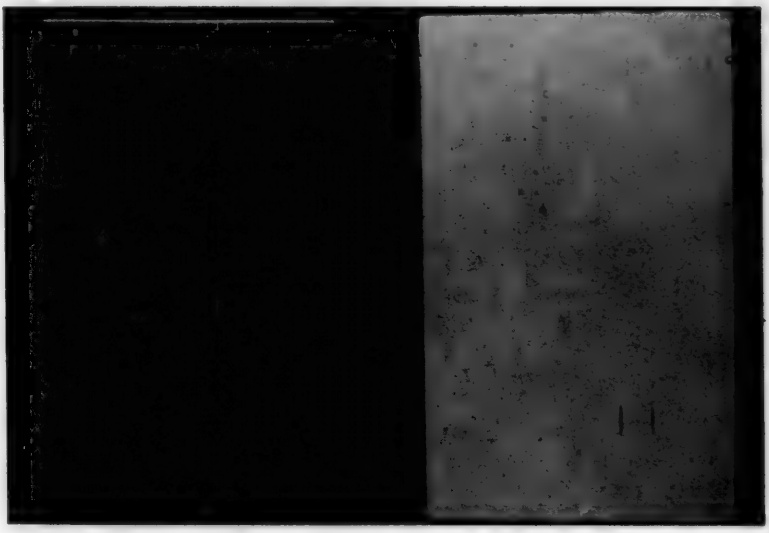

Fig. 1.—The original plate with fine lines (*left*) and a resultant stone model (*right*).

As separating mediums on the lined plate, a very thin coating of petroleum jelly was used for the alginate impressions, and a mix of Magicote (2 parts), a surface tension reducing agent (1 part), and distilled water (3 parts) was spread very thin for the mercaptan rubber-base impressions.

An impression was made of this plate, keeping a 3 mm. thickness of impression material, at a room temperature of 17° to 20° C.

All impression materials were mixed as directed by the manufacturers. Alginate impressions were removed from the original model 7 minutes after application, and poured immediately or after an immediate fixing. Before pouring, the excess fixing solution was removed by one or two gentle shakes of the impression to an extent where the impression surface was still wet and

glossy with a thin film of fixing solution on the alginate surface. Leaving excess solution fluid or drying off with an air syringe roughened the die surface.

Mercaptan rubber-base impressions were removed from the original model after 20 minutes and poured immediately.

The poured impressions were kept in a humidor at 100 per cent relative humidity. The stone models were removed from the impression after an hour.

A microscope which magnified 40 times and an oblique illumination was used to examine the reproduced lines and surfaces of the stone models.

RESULTS

The results were summarized in tables, in which the width of the finest line reproduced on the stone models from each group was compared in microns. So the smaller figure means the better result. The symbol x indicates the poorest results; those failing to reproduce even the 40 micron line. Four specimens were examined from each group, and no remarkable deviation was recognized in any group.

1. The influence of fixing conditions on the stone model surface from Alginate 1 impressions:

Two fixing mediums with varied concentrations and fixing times showed detail reproduction of Alginate 1 impressions as shown in Table II.

TABLE II
THE FINEST LINES REPRODUCED BY ALGINATE 1 IMPRESSIONS FIXED UNDER VARYING CONDITIONS

FIXING			POURED STONE			
SOLUTION	CONCENTR. (%)	TIME (SEC.)	HYDROCAL 1 (μ)	HYDROCAL 2 (μ)	DENSITE 1 (μ)	SURFACE
$ZnSO_4$	0.5	30	10	10	40	Excellent
		60	10	10	40	
	1.0	30	10	10	30	
		60	10	10	30	
	2.0	30	10	20	30	
		60	10	20	30	
	3.0	30	20	20	40	
	5.0	30	20	20	40	
H_2SO_4	0.1 N	30	10	20	30	
		60	20	20	30	
No fixing			30	30	40	

The 10 micron line could be reproduced by fixing impressions in a 0.5 to 2.0 per cent $ZnSO_4$ solution for 30 to 60 seconds when poured with Hydrocal 1 or 2. The smoothest model surface was observed under a microscope in the group fixed in a 1.0 per cent $ZnSO_4$ solution for 30 seconds. Increasing the concentration of the fixing solution beyond 2 per cent apparently roughened the model surface. Increasing the fixing time also seemed to roughen the model surface slightly.

Fixing in a 0.1 N H_2SO4 solution also reproduced the 10 micron line, but increasing the fixing time roughened the surface.

The finest reproduced line without fixing was 30 microns.

Densite type stone generally produced a poor surface with the alginate impression.

2. The influence of fixing conditions on the model surfaces from various alginate impressions:

Throughout all brands of alginates and stones tested, the finest line was reproduced by fixing impressions in a 1 per cent $ZnSO_4$ solution for 30 seconds, as shown in Table III.

TABLE III
THE FINEST LINES REPRODUCED BY VARIOUS ALGINATE IMPRESSIONS FIXED UNDER VARYING CONDITIONS

IMPRESSION MATERIALS	FIXING			POURED STONE		
	SOLUTION	CONCENTR. (%)	TIME (SEC.)	HYDROCAL 1 (μ)	HYDROCAL 2 (μ)	DENSITE 1 (μ)
Alginate 1	$ZnSO_4$	1	30	10	10	30
			60	10	10	30
		2	60	10	20	30
		no fixing		30	30	40
Alginate 2	$ZnSO_4$	1	30	40	40	x
			60	x	x	x
		2	60	x	x	x
		no fixing		x	x	x
Alginate 3	$ZnSO_4$	1	30	30	40	x
			60	40	40	x
		2	60	40	40	x
		no fixing		x	x	x
Alginate 4	$ZnSO_4$	1	30	30	30	40
			60	30	30	40
		2	60	30	40	40
		no fixing		40	40	x
Alginate 5	$ZnSO_4$	1	30	10	10	30
			60	10	10	30
		2	60	20	20	30
		no fixing		30	30	30

3. Comparison of the surface reproduction of various alginate and mercaptan rubber-base impression materials and stones:

The results in the best conditions from various impression materials and stones are compared in Table IV.

TABLE IV
COMPARISON OF THE FINEST LINES REPRODUCED BY VARIOUS ALGINATE AND MERCAPTAN RUBBER-BASE IMPRESSIONS AND VARIOUS STONES

IMPRESSION MATERIALS	POURED STONES			
	HYDROCAL 1 (μ)	HYDROCAL 2 (μ)	HYDROCAL 3 (μ)	DENSITE 1 (μ)
Alginate 1	10	10	x	30
Alginate 2	40	40		x
Alginate 3	30	40	40	x
Alginate 4	30	30	x	40
Alginate 5	10	10	x	30
Mercaptan 1	10	10	10	10
Marcaptan 2	10	10	10	10

Mercaptan rubber-base impressions reproduced the 10 micron line with any type of stone but failed to reproduce the 8 micron line, although it was reproduced on the impression surfaces.

Alginates 1 and 5 impressions also could reproduce the 10 micron line with Hydrocal 1 or 2 and failed to reproduce the 8 micron line. Alginate 1 produced a slightly smoother model surface than Alginate 5 when examined under a microscope.

Hydrocal 1 and 2 produced, respectively, the best and the second best surfaces of all the impression materials tested. Hydrocal 3 and Densite 1 produced a poorer die surface in all alginate impressions. Hydrocal 3 produced, however, a slightly better surface with Alginate 3 than with Alginate 1, in contrast to the other stones.

The photographs of reproduced lines of representative specimens are compared in Fig. 2.

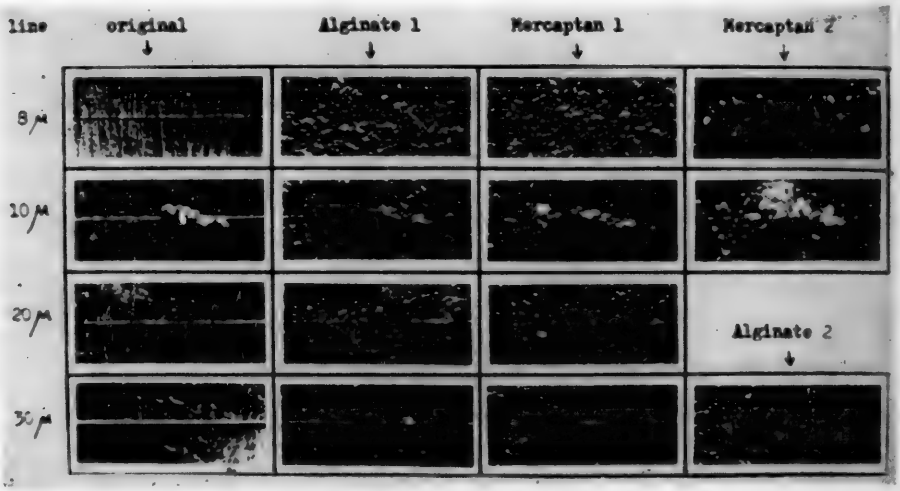

Fig. 2.—Comparison of the original lines and the lines reproduced on the resultant models of Hydrocal 1.

Consideration of the Model Surfaces From Alginate Impressions.—

Skinner[4,5] reported that the solutions of metallic sulfates were the most suitable for fixing alginate impressions. Fusayama[6] found the best fixing effect with a zinc sulfate solution for a Technicol (Alginate 1) impression.

In this study, the best result was obtained by fixing the Alginate 1 impression in a 1 per cent $ZnSO_4$ solution for 30 seconds. This fixing method showed the best results also of all other alginates tested. Increasing its concentration over 3 per cent apparently roughened the model surface, but the use of a 2 per cent $ZnSO_4$ solution in the clinic may be a practical idea since its concentration will be decreased by a repeated use which is common in practice.

Fixing time of 30 seconds is considered optimum because increasing the time seemed to roughen the model surface slightly and longer fixing time possibly increases the distortion of the impression.[7]

As stated in procedure, the excess fixing solution should be removed by a light shake of the impression leaving a wet glossy film of solution on its surface before pouring the stone. Leaving excess fixing solution fluid in the impression or drying it too much with an air syringe damaged the model surface.

Fixing alginate in a 0.1 N H_2SO_4 solution was suggested by Yamaga.[8] This method produced good surface detail. It is, however, not recommended in the clinic since it is rather critical and is liable to cause a shrinkage of alginate (the data will be reported in another paper).

All unfixed alginate impressions produced much worse model surfaces than surfaces produced by fixed impressions, although the manufacturers of those materials directed no fixing. Thus, fixing of alginate impressions is essential, regardless of the manufacturer's direction, for indirect inlay and crown technic, although it might not be required for denture work.

Alginate 5 produced a good model surface, nearly comparable to that of Alginate 1, but proportioning the paste and plaster or making a homogeneous mix may be rather difficult in the clinic since this material is a paste type to be mixed with plaster powder.

Densite 1 produced the poorest model surface in alginate impressions. This looks like a common characteristic of densite type stones since the same results were observed with several American alginates and densites by Fusayama when he was in the United States.

Consequently, hydrocal-type stones should be poured in alginate impressions. With alginates, Hydrocal 1 and 2 produced a much better surface than Hydrocal 3. Hydrocals 1 and 2 were made in Japan, and considered comparatively pure hydrocals because its setting expansion was higher than 0.5 per cent. This compares to that of pure hydrocal determined by Hollenback.[9]

On the other hand, the setting expansion of Hydrocal 3, made in the U.S.A., was lower than 0.2 per cent. Bright small crystals were found on its rough model surface. Thus, it seemed to contain a greater amount of substance for inhibiting the setting expansion. This chemical might roughen the model surface. The higher setting expansion of a model stone for indirect purposes is not always considered a disadvantage but an advantage because it compensates for the lack of investment expansion, as reported by Fusayama.[10, 11] Thus, a rather pure hydrocal is considered preferable for an alginate impression.

Hydrocal 3 produced a better surface with Alginate 3 than with Alginate 1, in contrast to the other stones. Mutual adaptation should be also considered in matching impression materials and stones.

In conclusion, the conditions required to obtain an excellent model surface with an alginate indirect technic is summarized with the following 6 factors:

1. An excellent alginate impression material (Alginate 1) should be used.
2. It should be spatulated as thoroughly as possible in one minute.[7]
3. The impression should be immediately fixed in a 1 to 2 per cent $ZnSO_4$ solution for 30 seconds.

4. The excess fixing solution should be removed by one or two gentle shakes of the impression, leaving a wet glossy surface, before pouring the stone.

5. A rather pure hydrocal-type stone should be used instead of a densite-type stone.

6. The model in the impression should be removed at least one hour after pouring.[12, 13]

Comparison of Mercaptan Rubber-Base and Alginate Impressions.—

The two mercaptan rubber-base impression materials tested produced excellent model surfaces, regardless of the brands of the poured stones. However, the surface reproduction of the alginate impression with the best materials and manipulation was as good as that of the mercaptan rubber-base impressions. They failed to reproduce the 8 micron line but reproduced the 10 micron line, which was much finer than the 40 micron line tested by Miller[3] to determine the suitability of elastic impression materials for use in an indirect technic.

SUMMARY AND CONCLUSION

The reproduction of the 8 to 40 micron lines by alginate and mercaptan rubber-base impressions on the stone models was investigated and the following results were found.

1. An alginate impression for indirect inlay and crown work should be fixed even if the manufacturer directed no fixing.

2. Fixing in a 1 per cent $ZnSO_4$ solution for 30 seconds produced the most excellent surface for any brand of alginate impressions tested.

3. Alginate 1 generally produced a better model surface than all other alginates tested when poured with most stones, while only Hydrocal 3 produced a little better surface with Alginate 3 than with Alginate 1.

4. In alginate impressions, a rather pure hydrocal-type stone is preferred to those stones containing more substances which inhibit setting expansion. A densite-type stone is contraindicated with alginate impressions.

5. The mercaptan rubber-base impressions produced good model surfaces from either hydrocal or densite stones.

6. Both alginate and mercaptan rubber-base impressions, used in the most ideal conditions described above, could reproduce the 10 micron line but both failed to reproduce the 8 micron line.

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FINE STRUCTURES OF THE DENTAL PULP

JAMES L. MATTHEWS, HOMER L. DORMAN, AND JACK G. BISHOP

From the Departments of Anatomy and Physiology, Baylor University College of Dentistry, and Baylor University Graduate Research Institute, Dallas, Texas

INTERRELATIONSHIPS of nerves and blood vessels can be studied in the dental pulp with little interference from other tissue elements. Pulp tissue contains numerous nerves and vessels of varying size suspended in a fibrous and sparsely cellular gelatinous matrix. Graf[1] made a quantitative study of the types of nerves in the human dental pulp and reported that a pulp cross section contains approximately one thousand separate nerve fibers, varying between 1 and 10 μ in diameter. The maximum size of arterioles in dental pulp has been measured by Provenza,[2] who reported diameters ranging up to 100 μ. The adventitia of the arterioles, metarterioles, and precapillaries were obliterated by dense bundles of nerve fibers and by the adjacent interstitial tissue. Smooth muscle innervation by autonomic fibers is generally described by light microscopists[3] as a plexus of unmyelinated nerves that terminate on the surface or within the sarcoplasm of the muscle fibers. Connections between the nervous and muscular elements in mammalian urinary bladder and in the ureters were examined with the electron microscope by Ceasar.[4] These neuromuscular structures consisted of simple contacts between the cytolemma of the smooth muscle and the lemnoblast of autonomic fibers. Smooth muscle tissue in these preparations was seen to be cellular and no evidence was obtained in support of a syncytial arrangement.

Electrophysiologic and pharmacologic data indicate the vasomotor role of the autonomic fibers,[5] but they do not indicate the morphologic basis for the vasodilator and vasoconstrictor mechanisms. Data have been collected in this laboratory which indicate the presence of sympathetic vasoconstrictor fibers in the mandibular nerve.[6, 7]

The present investigation utilizes the electron microscope for the study of structures in the dental pulp and particularly emphasizes the blood vessel nerve complex. The fine structure of all vessel and nerve types was examined in an attempt to define the structures which mediate vasomotor tone.

METHODS

Intact pulp specimens were obtained by cleaving cuspid teeth of anesthetized mongrel dogs. The specimens, obtained from apical, coronal, and

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medial regions of the dental pulp, were fixed for 30 minutes in 2 per cent osmic acid and were subsequently embedded in methylmethacrylate and butylmethacrylate (9:1) in a routine manner. The blocks were cut with glass knives on a Porter-Blum microtome. Orientation of the specimens was performed with the light microscope, using sections, 1 μ thick, cut from the same specimen block from which ultrathin sections were taken for electron microscope examination.

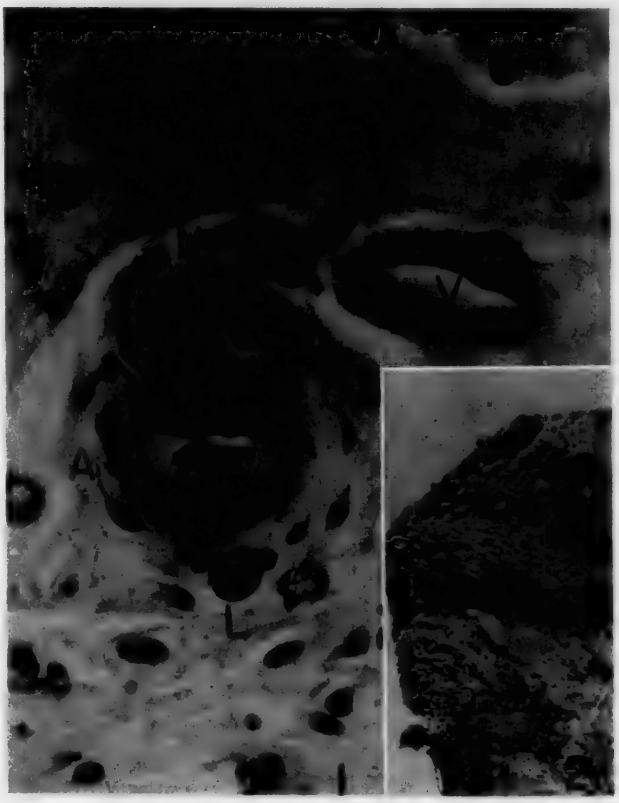


Fig. 1.—Section through osmium-fixed, methacrylate-embedded pulp cut 1 μ thick (orig. mag. ×600). *V*—venule; *N*—myelinated nerve; *C*—capillary; *A*—arteriole; *L*—lymphatic.
Fig. 2.—Pulp section, as it appears in the low-power light microscope (orig. mag. ×100).

Sections for light microscopy were prepared and stained according to the Runge method.[8] The ultrathin sections were observed with the RCA EMU-2B microscope.

RESULTS

Light microscope studies proved inadequate to resolve the neuromuscular connections to the blood vessels but were useful in obtaining a perspective of the distribution of the pulpal structures. Arterioles, venules, capillaries, lymph vessels, and myelinated nerves were often found closely grouped into bundles and surrounded by connective tissue fibers (Figs. 1 and 2). Electron microscopy demonstrated smaller unmyelinated fibers in areas of the blood vessels and

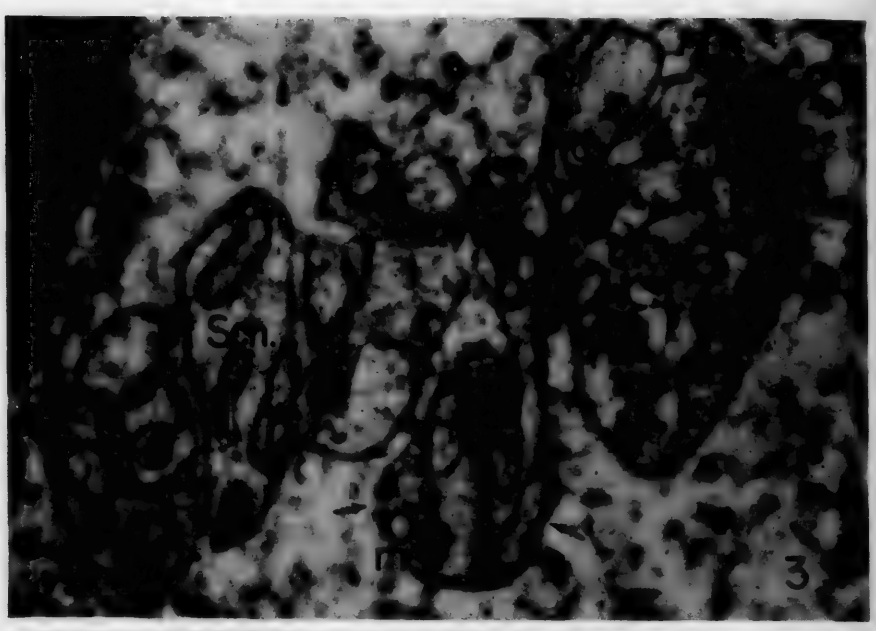
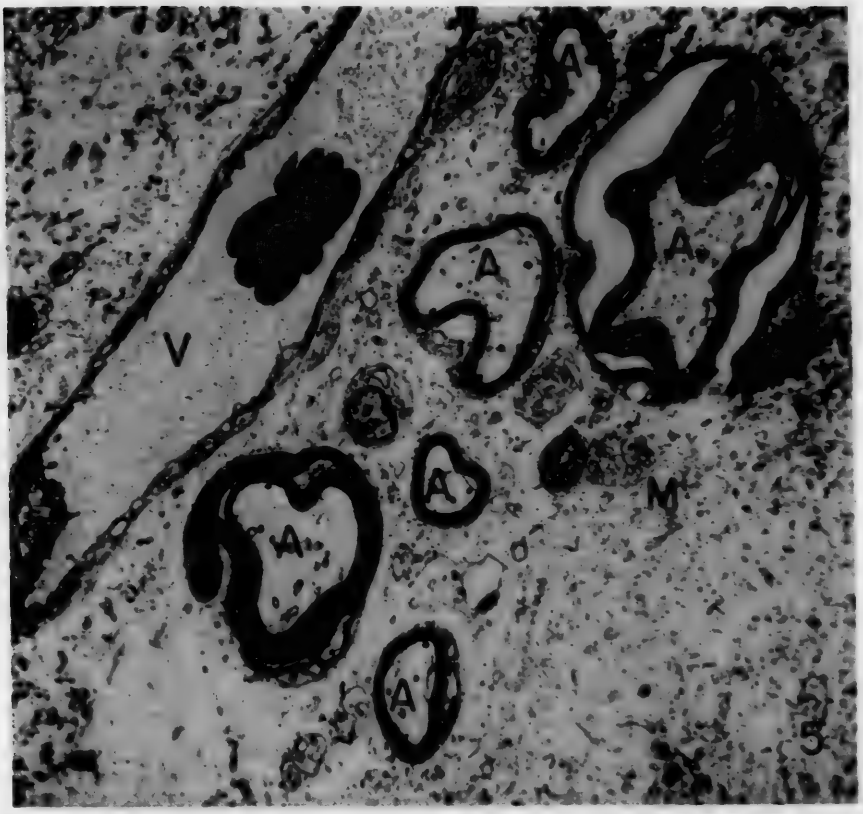


Fig. 3.—Unmyelinated nerve group (orig. mag. ×22,000). *C*—capillary; *Sch*—Schwann cell; *a*—axon; *m*—mesaxon arrangement (*arrows*).

Fig. 4.—Nerve fiber groups near a venule (orig. mag. ×3,100). *M*—mesaxon arrangement; *SN*—Schwann cell nucleus; *L*—lymphatic; *A*—axon; *V*—venule.



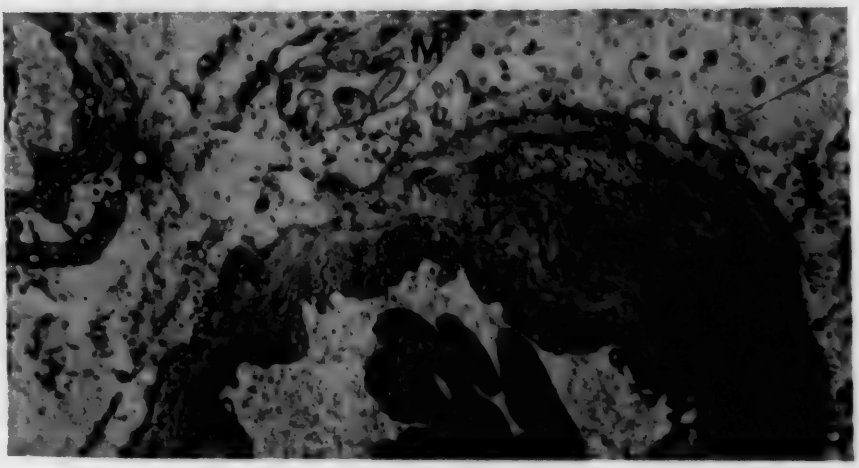


Fig. 5.—Section through the region of a large venule and large myelinated nerves (orig. mag. ×3,100). *M*—mesaxon arrangement; *A*—axon; *V*—venule.

Fig. 6.—Arteriole with a mesaxon arrangement near the sarcolemma of the smooth muscle (orig. mag. ×10,200). *SM*—smooth muscle; *M*—mesaxon.

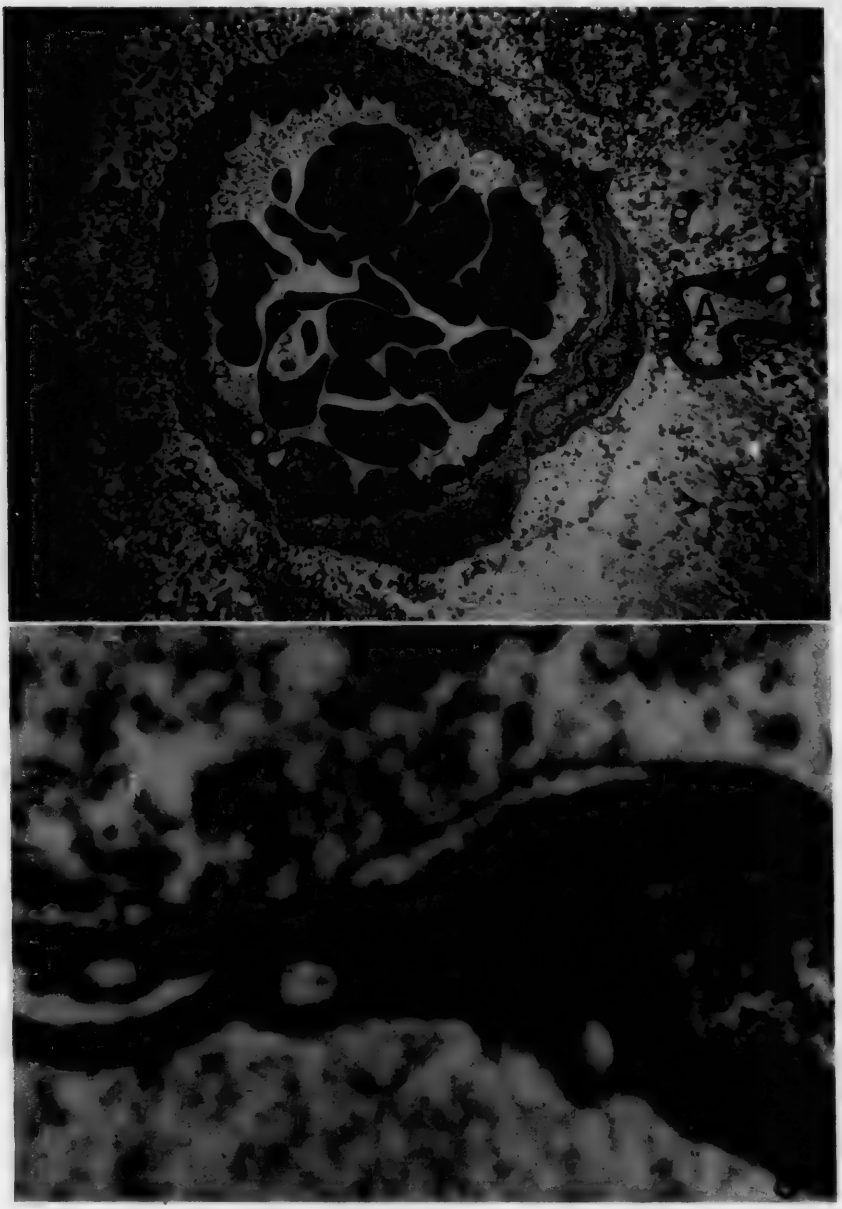


Fig. 7.—Entire arteriole showing *M*—mesaxon arrangement; *A*—axon of myelinated nerve fiber; *F*—fibroblasts; and *SM*—smooth muscle (orig. mag. ×3,100).

Fig. 8.—Capillary endothelial cell junction (*arrows*). *N*—nucleus of endothelial cell (orig. mag. ×22,000).

myelinated nerve fibers. Individual nonmyelinated fibers were found throughout the pulp but were more abundant in the areas of the larger vessels. Representative pulp nerve components are shown in Figs. 3, 4, and 5. Single myelinated fibers with diameters of 10 to 20 μ are present. The axons of each fiber are ensheathed in a Schwann cell. The cytoplasm of the Schwann cell is evident around all myelinated axons and the nucleus is observable in several. The maximum myelin thickness approaches 1.5 μ and is comparable to the A-alpha fibers of other peripheral nerves. The sheath cell membrane is a double-walled structure with a thickness of 10 mμ. A second nerve type, which is smaller than the myelinated fibers and is composed of several axons ensheathed in a common Schwann cell (Fig. 4), was observed. Only an occasional Schwann cell nucleus is seen in this group; the cytoplasm apparently extends for longer lengths along the smaller axon fibers. Folds of the plasma membrane enclose the peripheral extensions of the neurons. The axons are thus suspended within the area enclosed by the infolded plasma membrane of the sheath cell. This overlapping arrangement of Schwann cell cytoplasm has been designated a mesaxon. Fig. 3 shows the fundamental details of this arrangement. Each axon is limited by its own membrane and is further enclosed in the folds of the sheath cell. A third type of nerve element is randomly distributed, where individual axons are present which are singly surrounded by the plasma cell membrane of the sheath cells. No neurofibrils or unsheathed axons were noted in the pulpal tissue. The nerve groups routinely found adjacent to the described vessels are the unmyelinated fibers sheathed in the mesaxon arrangement.

The arterioles and venules of the pulp did not exceed 100 μ in diameter. Several vessels of smaller size were demonstrated. The walls of the arterioles (Figs. 6 and 7) were thin and the tunica adventitia of all vessels was not distinctly separated from the surrounding connective tissue matrix. The tunica intima consisted of a single elastic lamina and endothelial cells. Cytoplasmic fragments containing distinct granules and mitochondria with intact plasma membranes were seen adjacent to the lumen surfaces of the endothelial cells of most of the arterioles and metarterioles (Fig. 7). The majority of the arteriolar vessels were limited to a single layer of muscle cells in the tunica media. These structures were designated metarterioles.

The capillaries (Fig. 8) consisted of endothelial cells loosely surrounded by connective tissue. Plasma membranes of adjacent endothelial cells were visible at their junction and were separated by a 10 mμ space. A flap-like extension of the cells into the lumen was a typical finding in all capillary membrane junctions.

DISCUSSION

Unmyelinated nerve fiber axons ensheathed in Schwann cell cytoplasm with a mesaxon arrangement were located close to the walls of arterioles (Fig. 6). It is tempting to view these structures as the functional myoneural junctions, since no other structures were found which could plausibly perform this function. The presence of unmyelinated complexes on arterioles is consistent with

the concept that the site of vasomotor regulation is the arteriole and metarteriole. The small distance between distinct plasma membrane of the smooth muscle cell and the sheath cell may be due to the inherent shrinking which occurs during tissue fixation. This is particularly true in the wall of a collapsed vessel whose contents have escaped. Although a specific contact structure could not be demonstrated, an increase in the mitochondria and vesicles of the smooth muscle cytoplasm in the regions adjacent to the axons was seen. If such axon-muscle contact regions are considered as the synapses, the contact membranes may serve the same function as skeletal muscle end plates.

The endothelial cells forming the inner border of the arterioles and metarterioles consistently appeared as granulated cytoplasmic structures. Few nuclei were seen in any one cross section. This suggests that the endothelial cells are long and narrow and are orientated parallel to the vessel axis.

SUMMARY

The fine structure of the dental pulp was studied with particular emphasis on the neuromuscular relationships. The finding of autonomic nerve fiber complexes abutting the smooth muscle cells of arterioles offer an histologic basis for transmission of sympathetic vasoconstrictor impulses to the vascular elements.

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THE IN VITRO EFFECTS OF ANTIBIOTICS ON ORAL SPIROCHETES

E. G. HAMPP* AND R. J. FITZGERALD

National Institute of Dental Research, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

IN an earlier paper, the authors reported the effects of a number of chemotherapeutic agents on the growth in vitro of the small oral treponemes and *Borrelia vincentii*.[1] Since that time the successful cultivation of *Borrelia buccalis* in vitro has been reported.[2] The purpose of this study was to compare the in vitro responses of these three types of oral spirochetes to some of the newer antibiotic agents.

METHODS

The strains of small oral treponemes and *B. vincentii* were the same as employed previously[1]; in addition, three strains of *B. buccalis* designated GT2, NH1, and 4-2 were tested.

The details of the assay procedure were essentially the same as reported previously[1, 2] with the following exceptions: drugs not sufficiently soluble in water were dissolved in absolute methanol or acetone, sterilized by filtration, and diluted with sterile distilled water to contain the desired final series of concentrations in 0.2 ml. This amount was added to each tube in the test series, following which 9.8 ml. of the freshly prepared growth medium, previously inoculated with a 3-day culture of the appropriate spirochete strain, was pipetted into each tube using aseptic precautions throughout. In the present study, the medium was enriched with 10 per cent canine ascitic fluid instead of the human serum albumin previously employed.[1] The medium used for *B. buccalis* received an additional supplement of 5 per cent ox serum concentrate.† The tubes, 15 by 85 mm. in size, were almost completely filled by the medium and were closed by No. 0 sterile rubber stoppers.

The inoculum for the tests was prepared by making appropriate dilutions in the test medium of 3-day-old cultures of the various strains of spirochetes grown in the 0.075 per cent agar-containing fluid medium. The cultures were thoroughly agitated to distribute the organisms uniformly throughout the media and microscopic counts were performed under dark-field illumination by a modified Petroff-Hauser technic using Breed pipettes, standard cover slips,

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*Senior Research Associate, American Dental Association, at the National Institute of Dental Research.

†Microbiological Associates, Inc., Bethesda, Maryland.

and calibrated microscopic lenses. In five separate experiments during the course of this study, the inoculum size per milliliter of the test medium averaged as follows: small oral treponemes, 2.0×10^6 (range $1.1\text{-}3.1 \times 10^6$); *B. vincentii*, 1.8×10^6 (range $1.5\text{-}2.6 \times 10^6$); *B. buccalis*, 0.5×10^6 (range $0.3\text{-}0.8 \times 10^6$).

Visual readings of the antibiotic titrations were made at 3, 5, and 7 days and compared with the control tube of each series for gross evidence of growth or inhibition of growth. The inhibitory end point was considered to be the lowest drug concentration at which no turbidity, or only a trace of turbidity, was visually evident when compared with the uninoculated control tube after incubation for 5 days at 35° C.

Between the eighth and eleventh days, samples of all tubes showing growth inhibition were examined by dark-field illumination to determine viability as evidenced by motility and morphologic characteristics of the spirochetes. For purposes of this study, the organisms were considered killed if motility was absent and numerous bizarre forms were found. This was considered to be a more exacting criterion of "spirocheticidal" activity since previous experience had revealed the presence of actively motile forms in tubes from which positive subcultures could not be obtained. The lowest drug concentration satisfying these requirements is referred to as the spirocheticidal end point.

Most of the tests were repeated, the first run usually employing fourfold drug dilution steps to ascertain the inhibitory range of the drug and the second run using twofold dilution steps to obtain a more precise end point.

RESULTS AND DISCUSSION

The in vitro effects of five antibiotic agents on the growth of three strains each of the small oral treponemes, *B. vincentii*, and *B. buccalis* are summarized in Table I. Spirocheticidal concentrations of the drugs determined by dark-field examination for morphologic integrity and motility are shown in parentheses in the same table.

The antibiotics listed are active primarily against gram-positive bacteria but it can be seen that they are all effective to some extent against the oral

TABLE I
INHIBITION OF ORAL SPIROCHETES IN VITRO

	SMALL ORAL TREPONEMES	*Borrelia vincentii*	*Borrelia buccalis*
Penicillin	0.005-0.01* (0.03)†	0.01-0.05 (0.02->1)	0.005-0.02 (0.02-0.05)
Oleandomycin	0.2-0.5 (0.6-5)	0.04-0.2 (0.1-5)	0.02-0.08 (0.03-0.1)
Novobiocin	0.3-6.0 (>40)	0.06-0.6 (0.3-40)	0.02-0.06 (0.06)
Celesticetin	5-10 (>40)	2.5-10 (>40)	2.5-5 (10)
Streptolydigin	0.6-1.2 (5-20)	0.6-1.2 (2.5->5)	0.3-2.5 (0.3-2.5)

*Inhibitory concentrations at 5 days, μg/ml.
†Spirocheticidal concentrations at 8-11 days, μg/ml.

spirochetes, which are gram-negative. In general, the degree of sensitivity of the spirochetes to these antibiotics is in about the same range as for the streptococci.[3, 4, 5] The greatest variation in response was encountered in the case of novobiocin. With this agent the growth inhibitory end point for the small oral treponemes ranged between 0.3 and 6.0 μg/ml. and the organisms were still viable at 40 μg/ml. The strains of *B. vincentii* were inhibited between 0.06 and 0.6 μg/ml. and killed between 0.3 and 40 μg/ml. whereas *B. buccalis* was inhibited between 0.02 and 0.06 μg/ml. and killed at 0.06 μg/ml.

Table II summarizes the results of tests with 5 additional antibiotic agents. Erythromycin, with an antibacterial spectrum similar to penicillin, is quantitatively the most effective drug in this group. Vancomycin, while less active, has the virtue of being able to kill bacteria and spirochetes at the same concentrations at which they are inhibited,[6] consequently there is less risk of the emergence of drug-resistant strains with this agent. Polymyxin B is primarily effective against gram-negative bacteria and it can be seen that it is relatively ineffective against the spirochetes, which seem to be similar to the gram-positive bacteria in their pattern of susceptibility to antibiotics. Cycloserine is effective against gram-positive, gram-negative, and acid-fast microorganisms in vitro, but only at comparatively high concentrations. It has been reported to be effective in *Borrelia novyi* infections in mice.[7] As shown in Table II, this drug also inhibits oral spirochetes in vitro only in relatively high concentrations.

TABLE II
INHIBITION OF ORAL SPIROCHETES IN VITRO

	SMALL ORAL TREPONEMES	*Borrelia vincentii*	*Borrelia buccalis*
Erythromycin	0.03* (0.03-2)†	0.008-0.015 (0.008->2)	0.004-0.008 (0.008-0.02)
Vancomycin	2.5 (2.5)	1.2-2.5 (1.2-2.5)	1.2-2.5 (1.2-2.5)
Polymyxin B	12-25 (>100)	12-50 (50-100)	12 (25-50)
Cycloserine	>100 (>100)	100->100 (>100)	50-100 (100->100)
Anisomycin	>100 (>100)	>100 (>100)	>100 (>100)

*Inhibitory concentration at 5 days, μg/ml.
†Spirocheticidal concentration at 8-11 days, μg/ml.

Anisomycin is an antibiotic with a rather limited spectrum of activity. So far as is known, it inhibits only the monilia and the trichomonads and endamoebae and is ineffective against bacteria.[8] It was also devoid of activity against the oral spirochetes in the present tests.

Berger[9] has independently tested various antibiotic agents in vitro against a series of the small oral treponemes isolated in Germany. In spite of the differences in media and technic, his results are in good agreement with those reported previously[1] and in the present work when comparisons of the same drugs could be made.

SUMMARY

A number of antibiotics have been tested in vitro against pure cultures of *Borrelia vincentii, Borrelia buccalis,* and the small oral treponemes. Penicillin, oleandomycin, novobiocin, celesticetin, streptolydigin, erythromycin, and vancomycin inhibited and killed these organisms at concentrations readily attainable in the oral cavity. The spirochetes were relatively resistant to polymyxin B and were markedly resistant to cycloserine and anisomycin.

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SALIVARY AND PLAQUE LACTOBACILLI IN THE PROGNOSIS OF HUMAN DENTAL CARIES

GORDON E. GREEN AND PAUL R. WEISENSTEIN

College of Dentistry, The Ohio State University, Columbus, Ohio

INTRODUCTION

PREVIOUS publications from this laboratory on the relation of oral lactobacilli and dental caries have been concerned with dental plaques (Green, Dodd, and Inverso[1]) and measurement of salivary lactobacilli with respect to caries prognosis in children (Green, Weisenstein, and Permar[2]). In the former paper, some results indicated that lactobacilli in dental plaques might be more closely related to the caries process than numbers of salivary lactobacilli. In one of these papers,[2] the results of other investigators in this area were reviewed.

The present investigation has been undertaken for the purpose of obtaining more extensive data on lactobacilli in saliva and dental plaques, and to relate these data, if possible, to current dental condition and future caries increment.

MATERIALS AND METHODS

Clinical Subjects.—The studies involved 107 members of the 1959 class of The Ohio State University College of Dentistry, who ranged in age from 20 to 38 years.

Clinical Examinations.—All dental examinations were made by the same dentist (P. W.) after a complete dental prophylaxis. The teeth were examined and charted with the aid of an adequate light, a mouth mirror, and an explorer. Bite-wing x-rays were provided in order to supplement diagnosis of interproximal lesions. Two dental examinations of the subjects were made one year apart.

Biologic Sampling.—Monthly samples were taken from each subject eleven times during the year of study, beginning with the initial examination. At each sampling, 10 ml. of paraffin-stimulated saliva were collected. Plaque material was collected after forceful rinsing of the tooth areas with a water spray. The material was removed with a scaler from no more than 2 interproximal areas of the most posterior teeth in each of the 4 quadrants, and from the lower anterior region. The material was forcibly scraped from the tooth surfaces in the interproximal area, approached from the buccal aspect. The plaque material was that remaining attached to the tooth surfaces (mesial or distal) below the

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contact point after forceful water washing. At each sampling, 1 saliva sample and 5 plaque samples were obtained from each subject who was available at the time. Some subjects missed a few samplings.

Bacteriologic Methods.—All samples were plated the day of receipt. Saliva samples were diluted in sterile saline and plated to give a 1:1,000 dilution on tomato juice agar at pH 5 containing bromcresol green and sodium azide (adapted from Hadley's medium[3]). Replicate plates were incubated aerobically and anaerobically for 4 days at 37° C. Saliva was also plated at 1:100,000 dilution on tryptone glucose extract agar (Difco) containing bromcresol purple indicator and 5 per cent $CaHPO_4$ (adapted from the medium of Davies, Slack, and Tilden[4]), for determination of acidogenic colonies. These plates were incubated aerobically for 48 hr. at 37° C.

Plaque samples were placed in a celluloid tube containing 10 ml. of sterile saline plus 0.05 per cent sorbitan monooleate.* The tube was positioned in the focal point of a hemispherical transducer driven by an ultrasonic generator.† The ultrasonic treatment was 3 min. at 25° C., with the power setting at 3. This treatment had previously been shown to be relatively harmless to the types of organisms under study.[2] After dispersion, the suspensions of plaque material were plated at 1:10 dilution on tomato juice agar and 1:1,000 dilution on tryptone glucose extract agar.

Colonies were counted with a stereoscopic microscope. Smooth, intermediate, and rough lactobacillus colonies were identified as previously described (Green and Dodd[5]).

Data Tabulation.—The data collected pertaining to each subject included the following: age, dentifrice usage, and diet habits. Bacteriologic data included, at each sampling, counts of lactobacillus colony types, of aciduric streptococci and micrococci, and acidogens in saliva, and in 5 plaque samples. Clinical data included DMFS score at first examination, decayed lesion scores at first examination, decay score increment in whole mouth and in quadrants, DMFS increments for the whole mouth and each quadrant.

TABLE I

CHARACTERISTICS OF THE 107 DENTAL STUDENTS EXAMINED

CHARACTERISTIC	RANGE	AVERAGE	MEDIAN
Age, years	20-38	21.3	22
Total DMFS score (1st exam.)	0-77	35.3	34.2
Decay score, unfilled lesions (1st exam.)	0-31	9.7	8.5
Decay score, unfilled lesions (2nd exam.)	0-31	10.5	9.2
DMFS increment during 1 year	0-18	3.9	2.6

The data were punched on IBM cards, 12 cards being required for each subject. Compilation of most of the data was accomplished by simple sorting into various categories. Summarizations of various groups of data were also punched in order to facilitate final tabulations.

*Tween 80.
†Rich-Roth 400.

RESULTS

Subject Characteristics.—Some characteristics of the population of subjects are shown in Table I. The clinical data show that this group was not receiving frequent extensive dental care, and lend justification to the use of these subjects for a caries study.

An interesting relationship between past and future caries history is shown in Table II. These data show a certain association between extent of past caries attack and severity of future attack up to some point where the number of surfaces remaining available for new caries is markedly reduced. In the entire group, however, the association was not statistically significant.

TABLE II
RELATION OF PAST CARIES HISTORY TO FUTURE CARIES INCREMENT

DMFS SCORE AT FIRST EXAM. (RANGE)	NUMBER OF SUBJECTS IN GROUPS	AVERAGE CARIES INCREMENT IN NEXT YEAR
0-9	6	0.8
10-19	13	3.7
20-29	22	3.3
30-39	29	4.6
40-49	15	5.0
50-59	10	3.7
60-70	10	3.5

Analysis (individual items, not ranges, were used): correlation coefficient $r = 0.096$; $t = 0.988$; d.f. $= 106$; $p = >0.3$ (not significant).

Salivary Flora and Dental Caries.—Comparisons were made between numbers of salivary lactobacilli counted at the first examination and the total DMFS scores at that time. The data in Table III show a relationship, statistically nonsignificant, which is even more distinct than was previously found in children.[2]

TABLE III
RELATION OF NUMBERS OF SALIVARY LACTOBACILLI AND DMFS HISTORY

DMFS SCORES (RANGE)	NO. OF PERSONS HAVING VARIOUS TOTAL LACTOBACILLUS COUNTS AT FIRST EXAM. LACTOBACILLUS COUNTS (CODED)*								
	0	1	2	3	4	5	6	7	8
0-5	2								
6-10	2	2							
11-15	2								
16-20	3	1	1					3	
21-25	2	2	1			3		3	
26-30	1		2	1		1	1	3	
31-35	7	3					1	3	
36-40		5		1		6		1	
41-45					1	6	2	1	
46-50					2		1	1	1
51-55	1				1	2	1	1	
56-60	1			1	1	1	1		
61-65	1				1	1		3	
66-70	1				1		2		
71-75									
76-80						1			

Analysis: correlation coefficient $r = 0.0138$; $t = 0.142$; d.f. $= 106$; $p = >0.5$ (not significant).

*1 = 0—499
2 = 500—999
3 = 1,000—4,999
4 = 5,000—9,999
5 = 10,000—49,999
6 = 50,000—99,999
7 = 100,000—999,999
8 = 1,000,000—50,000,000
9 = more than 50 million

Similar results were recently reported by Rovelstad, Geller, and Cohen.[6] Breakdown of total lactobacillus counts into counts of various colony types showed no closer degree of association.

Counts of total number of lactobacilli and counts of colony types were compared with the number of unfilled carious lesions in each subject. This was done at the beginning and at the end of the study year. No relationship was found.

Comparisons were made between numbers of certain members of the oral flora determined at the initial laboratory examination with the increase in DMFS score (caries increment) found at the end of 1 year. Table IV shows the relation of the total lactobacillus counts at the first examination to caries increments during the 1-year period. No association is evident. Counts made at the 6-month examination showed no closer relation to caries increment.

TABLE IV

RELATION OF TOTAL SALIVARY LACTOBACILLUS COUNTS AT FIRST EXAMINATION TO CARIES INCREMENTS DURING FOLLOWING YEAR

DMFS INCREMENTS	NO. OF PERSONS HAVING VARIOUS TOTAL COUNTS AT FIRST EXAM. LACTOBACILLUS COUNTS (CODED)*							
	0	1	2	3	4	5	6	7
0	1	1		1	1	2	1	1
1	3	3	1		1	3	1	5
2	5	1	2			4	2	
3	3	3			3	4	1	4
4	2				1	3		3
5	5			1	1	2	3	2
6	2	2		1		3	2	2
7			1			1		2
8		1				1	1	
9	1	2						1
10								1
14							1	1
18	1							

Analysis: correlation coefficient $r = 0.099$; $t = 1.027$; d.f. $= 106$; $p = >0.2$ (not significant).

*See Table III.

The average salivary lactobacillus count was determined for each individual for the first 6 months of the study and compared with the year's caries increment. The results again showed no association.

The lactobacillus counts made during the first 6 months of the study were scanned, and it was noted whether the counts tended to increase, decrease, or to remain constant during the period. The persons whose counts increased during the period had no higher average caries increment than the persons whose counts decreased. On the contrary, the reverse was true; for persons whose count increased, the average increment was 3.5, whereas those whose count decreased showed an average increment of 5.7. Analysis of the data in this case for the probability that the proportions expressed by the 2 groups were not equal gave these results: $t = 2.21$, d.f.) 36, $p = 0.05$, indicating the groups were significantly different. Breakdown of the total counts into numbers of smooth, intermediate, and rough colonies showed no closer association with future caries increment.

The relation of occurrence of aciduric streptococci and micrococci to caries increment was also studied. Again, computing the average number of organisms found in the first 6 examinations, those persons whose saliva showed only a few aciduric streptococci developing on tomato agar had a higher average caries increment (5.5) than those whose saliva showed many aciduric streptococci (4.0). Those having few aciduric micrococci had a higher average caries increment (3.6) than those having many micrococci (3.0). This observation could be due to the fact that, when many lactobacilli appear on a tomato agar plate, usually very few streptococci and micrococci are seen. The caries-immune group at Ohio State University usually showed large numbers of aciduric streptococci (and few lactobacilli).[5]

Counts of acidogenic organisms in saliva were made on tryptone glucose extract agar plus bromcresol purple indicator. Nearly all saliva samples contained large numbers of these organisms. No relationship was found between numbers of acidogens and future caries increment in individuals.

Relationship of Salivary and Plaque Lactobacilli.—The dental plaque is a relatively protected aggregation of bacterial cells which presents an environment which is in many ways quite different from that of liquid saliva. This subject has been discussed by many workers. Among recent reports, Mills[7] has shown marked differences in lactobacillus presence in saliva and plaques in a study in which quantitation was attempted. There is a possibility, however, that salivary and plaque microflora might resemble each other, not in number, but qualitatively, to some extent because the plaque is, to a great extent, deposited from the saliva and the plaque probably sheds organisms into the saliva. Examination of this thesis has been made because many bacteriologic examinations of saliva have been used, based upon the premise of similarity of salivary and plaque flora. Again, it should be stressed that the plaque is the engine of enamel destruction, and determination of plaque activities made by study of saliva should be based on known similarities or points of reference.

The occurrence of plaque-saliva resemblances is shown in Table V. The number of individuals in Group 1 (where usually lactobacilli were found in saliva and not in plaques rather than vice versa) increased during the year, as did the number in Group 3, having no lactobacilli at all. Since the number of

TABLE V

OCCURRENCE OF PLAQUE-SALIVA RESEMBLANCES IN PRESENCE OR ABSENCE OF LACTOBACILLI DURING THE YEAR OF STUDY

GROUP	PLAQUE-SALIVA RELATIONSHIP	NO. OF PERSONS IN WHOM RESEMBLANCE WAS FOUND DURING:	
		FIRST 6 MO.	SECOND 6 MO.
1	No resemblance	49	61
2	Positive (lactobacilli present in saliva and one or more plaque areas)	55	34
3	Negative (lactobacilli absent from saliva and all plaque areas)	2	11

persons in Group 2 decreased, the data indicate the frequency of lactobacillus-positive plaques decreased during the year. An explanation is not readily available, but it may be possible that the operations of the study had some direct or indirect effect on the degree of oral cleanliness in the subjects. The lactobacillus colony types (smooth, intermediate, and rough) were found to occur in salivas with frequencies approximating a 3:1:4 ratio. When compilation was made of the number of times that colony types occurred in saliva and one or more plaques in the same mouth, the frequency ratio was 3:1:4. This indicates that the rough colony lactobacilli were more apt to be found in both saliva and plaque than other types.

The relationship of plaque-saliva similarity to dental caries increment was also considered. It was found that those persons who had lactobacilli in both saliva and plaque had a slightly higher average caries increment than those who had lactobacilli in either or neither area.

The relation of lactobacilli in plaques to caries increments is shown in Table VI, along with pertinent statistical analyses. These data show that the subjects in Group E, having four or more lactobacillus-positive plaques during the year, had significantly higher caries increments (averaged) than subjects having no positive plaques. It is clear that lactobacilli in plaques were somehow associated with caries processes, yet these findings could not be used to predict individual caries rates. Along this line, Hemmens and co-workers[8] have stated, "It should be noted that in every instance in which lactobacilli were recovered from a plaque from a noncarious area there was active caries elsewhere in the mouth."

TABLE VI

RELATION OF OCCURRENCE OF LACTOBACILLI IN PLAQUES TO DMFS INCREMENTS

GROUP	NO. OF LACTOBACILLI—POSITIVE PLAQUES DURING: FIRST 6 MO.	SECOND 6 MO.	NO. OF SUBJECTS	AVERAGE DMFS INCREMENT
A	0	0	38	3.61
B	only one during year		22	3.09
C	>1	0	16	3.63
D	0	>1	9	4.11
E	>1	>1	22	5.64

Analysis: "t" test for significant difference between the means of groups:

A and E: $t = 2.09$, $p = <0.05$ significant
B and E: $t = 4.59$, $p = <0.01$ significant
C and E: $t = 1.90$, $p = 0.06$ nonsignificant
D and E: $t = 1.15$, $p = 0.26$ nonsignificant
A and D: $t = 0.49$, $p = >0.50$ nonsignificant
C and D: $t = 0.50$, $p = >0.50$ nonsignificant

Observation of the occurrence of aciduric streptococci and micrococci in plaque samples showed that persons having aciduric streptococci had an average caries increment of 4.5. These figures are slightly higher than the average increment for the whole group (3.9), but probably are not significant. The finding is the reverse of the association described in saliva and will be further studied as it may indicate the presence of a plaque mechanism not reflected in saliva.

The occurrence of acidogenic organisms was not directly associated with above-average caries increments.

Compilations of data were made to determine whether presence of lactobacilli in a specific plaque area could be associated with the presence of open lesions in that quadrant and the occurrence of new lesions in the quadrant. Lactobacilli in plaques were found more frequently when carious lesions were continuously present in the quadrant than when lesions were present only at the first or second clinical examination or neither. However, the occurrence of lactobacilli in a plaque area could not be correlated with whether or not a caries increment was found in that quadrant, as is shown in Table VII. The differences are not consistent and may not be significant.

TABLE VII

RELATION OF FREQUENCY OF OCCURRENCE OF LACTOBACILLI IN PLAQUE SAMPLES FROM ONE AREA TO DENTAL CARIES INCREMENT IN THAT QUADRANT

PLAQUE AREA (QUADRANT)	AVERAGE CARIES INCREMENT IN GROUPS			
	FIRST 6 MO. (6 EXAM.)		SECOND 6 MO. (5 EXAM.)	
	LACTOBACILLI PRESENT (0 OR 1 TIME)	LACTOBACILLI PRESENT (2 OR MORE TIMES)	LACTOBACILLI PRESENT (0 OR 1 TIME)	LACTOBACILLI PRESENT (2 OR MORE TIMES)
Right upper	0.55	0.54	0.56	0.50
Right lower	0.51	0.62	0.51	—*
Left upper	0.84	0.58	0.80	1.00
Left lower	0.55	1.11	0.52	1.00
Lower anterior	—†	—	—	—

*Only one person in this group.
†Infrequent occurrence of lactobacilli, no comparison made.

This work was designed to test the possibility that caries activity might be reflected in general changes in the lactobacillus population of dental plaques; that is, that many plaques in a caries-active mouth might show a changed cariogenic potential even on surfaces which would not shortly become carious. This possibility was considered because of the obvious advantages which would result if one were able to predict caries activity or inactivity based on examination of only a few plaque samples. Comparison of the data in Table VII with those in Table VI shows that while the frequent presence of lactobacilli in plaques may be loosely related to caries activity, such presence is not a satisfactory indication of caries activity in the area immediately adjacent to the tooth surface from which plaques were taken.

Summary of Comparisons of Oral Microflora With Dental Caries.—

1. *Relationship to caries history (DMFS score):*

Total number of salivary lactobacilli—no association.
Number of smooth colony lactobacilli—no association.
Number of intermediate colony lactobacilli—no association.
Number of rough colony lactobacilli—absent in persons with DMFS score below 15.

2. *Relationship to number of current open cavities (decay score):*

Number of smooth colony lactobacilli—no association.
Number of intermediate colony lactobacilli—no association.
Number of rough colony lactobacilli—no association.

3. *Relationship to future caries increment:*

Total number of lactobacilli (first exam.)—no association.
Average number of lactobacilli (first 6 exam.)—no association.
Increase and decrease in total count during first 6 exam.—inversely related.
Number of smooth colony lactobacilli—no association.
Number of intermediate colony lactobacilli—no association.
Number of rough colony lactobacilli—no association.
Number of aciduric streptococci—inversely related.
Number of aciduric micrococci—inversely related.
Number of acidogenic organisms—no association.

4. *Saliva-plaque relationships:*

Chances for similarity and dissimilarity in presence of lactobacilli were about equal.
Of the three lactobacillus colony types, the rough type was found simultaneously in saliva and plaque with greatest frequency.
Persons having lactobacilli in both saliva and plaque had a slightly higher caries rate (average) than persons who did not have lactobacilli in both areas.

5. *Relation of plaque microflora to dental caries:*

Presence of lactobacilli in four or more plaque areas during the study year was associated with higher caries increment (group average) than found in persons not having plaque lactobacilli.
Presence of aciduric streptococci or micrococci was associated with a slightly higher average caries increment than found in the whole group. Presence of acidogenic organisms was not associated with dental caries increment. Presence of lactobacilli in at least one plaque sample from an area was associated with open lesions in that quadrant, but not with caries increment in the quadrant.

DISCUSSION

This investigation has attempted to provide additional information on the relation of oral lactobacilli to human dental caries. Some limitations of the methods employed are obvious: the limited number of samples and the oft-forgotten fact that counts of salivary lactobacilli refer only to the number of colonies which grow on a particular medium. It is almost certain that the counting methods currently in use can only estimate the number of viable oral lactobacilli.

One main objective of this work was to continue the study of the relation of salivary lactobacilli to future dental caries in a different age group from the group described in a previous report.[2] In this present work, it was found that numbers of salivary lactobacilli were as unsatisfactory for predicting future dental caries in young adults as had been found in 6- to 14-year-old children.

Tests for caries activity such as lactobacillus counts and the Snyder colorimetric test[9] were based on the assumption that the presence of caries-producing mechanisms is reflected in general changes in the salivary flora. A similar assumption is that caries-producing mechanisms might effect general changes in most dental plaques within a given mouth. Stephan[10] has shown that plaques in caries-active mouths are capable of lower pH minima than plaques in caries-inactive mouths. Also, Stralfors[11] has shown that pH minima in plaques may be correlated with numbers of salivary lactobacilli. In a brief report, Hemmens, Blayney, and Harrison[12] indicated that the difference between lactobacillus composition of saliva and plaques was of a quantitative, not qualitative, nature. The work of Mills[7] generally confirms this; however, Krasse,[13] among others, has pointed out that many instances of apparent qualitative difference may occur in individual mouths. There is sufficient leeway for different interpretations of the findings of various writers that the relationship of lactobacilli to dental caries is quite unclear.

Hemmens and her co-workers[8, 12, 14, 15, 16] have conducted a most detailed series of studies of the flora of dental plaques in relation to caries. Their work has shown a number of changes in plaque flora prior to the appearance of clinically detectable carious lesions. Most of their work has dealt with the changes in single tooth surfaces related to the plaques removed from the specific surfaces. The most outstanding finding was the increased incidence of lactobacilli in precarious plaques compared to plaques from surfaces that did not become carious.

The second major objective in this work was to test the possibility that the presence of caries-producing mechanisms in a single mouth might be reflected in obvious changes in the flora of many plaques in that mouth. No attempt was made to correlate plaque conditions with events on a single tooth surface. The purpose was rather to determine if recently developed technical methods of plaque study[1] might detect general changes in plaque flora which would serve as indicators of future dental caries. The results obtained have, of course, confirmed the work of other investigators; that is, an increased incidence of lactobacilli may be found in most persons in whom caries are active in comparison to noncaries-active persons. The relationships found are sufficiently general that they do not permit accurate prediction of future caries rates in single individuals. It is obvious that observations of only lactobacilli, whether in saliva or plaques or both, cannot produce enough evidence to warrant accurate statements regarding caries activity in individual human beings.

SUMMARY

Lactobacilli and certain other microorganisms were counted in saliva and plaque samples taken at monthly intervals from 107 dental students. Samplings were made during one year, at the beginning and end of which complete dental examinations were made. Comparisons were made between numbers of various organisms and previous caries attack, current dental condition, and future dental caries increment. Some associations were found, none of which, however, was

clear and consistent enough to permit accurate prognosis of future dental caries in individuals. Certain relationships between salivary and dental plaque microflora are described.

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HISTOCHEMICAL LOCALIZATION OF HYDROLYTIC ENZYMES IN HUMAN SALIVARY GLANDS

HOWARD H. CHAUNCEY AND GIULIANO QUINTARELLI*

Department of Dental Research, Tufts University School of Dental Medicine, Boston, Mass.

AVAILABILITY of methods for the rapid collection of uncontaminated human saliva greatly facilitate the study of salivary gland function. Accordingly, the chemistry of these secretions and metabolic status of the glands can be readily ascertained, whereas other human glands are less accessible. Although several previous investigations[1-5] have indicated that the enzyme and electrolyte composition of saliva are influenced by various local and systemic factors, there is little information available concerning the intraglandular origin of the hydrolytic enzymes present in saliva.

The current study was undertaken in an effort to determine the localization of acid phosphatase, nonspecific esterases, and β-D-galactosidase in the various cells comprising the parotid and submaxillary glands. In addition, it was desired to evaluate reaction intensity differences between the two glands and determine the effect of subject age on enzyme reactivity.

METHOD

Collection and Storage of Tissues.—Specimens from 22 persons of both sexes were examined (5 children, 8 young adults, and 9 elderly persons). The adult material was obtained from persons whose death required prompt autopsy by a legal examiner. Thus, the glands were usually removed within ½ to 1 hour, but no later than 5 hours, following clinical death. Most of the subjects were victims of accidents who otherwise appeared to be of normal health. Tissues which showed obvious pathology were not used. Specimens were stored at −10° C. until the histochemical procedures could be initiated. Subsequent histologic examination revealed that all samples employed were without signs of cellular degeneration. Parotid and submaxillary glands were routinely removed. The sublingual gland could not be obtained in most instances because removal was likely to cause disfigurement. Thus, the histochemical description contained herein is limited to the former two glands.

Sectioning and Mounting.—Tissue samples were thawed as desired and cut into blocks approximately 2 to 3 mm. thick. Fixation was for 18 to 24 hours

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*Present address: University of Alabama, School of Dentistry, Birmingham, Ala.

at 4° C. with chloral-hydrate-formal (CHF) solution. After fixation, the tissues were washed in running tap water for 2 hours. Salivary gland tissue was found to be extremely friable when cut on a freezing microtome. This condition was alleviated by embedding the tissue in gelatin, using the technic described by Fishman and Baker.[6] Following localization of hydrolytic enzyme activity the sections were mounted on glass slides with glychrogel. The mounting medium was prepared by dissolving 100 mg. of potassium chrome alum in 15 ml. of distilled water. One and one half grams of gelatin was dissolved in 25 ml. of hot distilled water and the two solutions were carefully mixed together, to avoid clumping of the gelatin. Ten milliliters of glycerin, one crystal of camphor, and one crystal of menthol were added, and the mixture was carefully stirred to ensure a homogenous composition. Finally, the pH was adjusted to 7.4 with solid sodium bicarbonate.

Histochemical Technics: Acid phosphatase and β-D-galactosidase were demonstrated using the procedure described by Rutenburg and Seligman[7] and Cohen, and his co-workers.[8] Esterase activity was localized using the basic method outlined by Gomori,[9, 10] with modifications as indicated.

Acid phosphatase: 25 mg. of sodium 6-benzoyl-2-naphthyl phosphate were dissolved in 100 ml. of 0.1 M acetate buffer, pH 5.0. In addition, each 100 ml. of buffered substrate contained 2.0 grams of sodium chloride, producing a hypertonic solution which aided in reducing diffusion of the enzyme into the substrate medium. CHF-fixed tissues were incubated for 20 to 30 minutes at 37° C. After incubation, the sections were washed in distilled water for several minutes. Enzyme activity was visualized by immersing the sections in a cold (4° C.), freshly prepared solution of diazotized-o-dianisidine* (1 mg./ml.) which had been alkalinized with sodium bicarbonate. One minute was allowed for the reaction between liberated 6-benzoyl-2-naphthol and the diazionium salt; which resulted in the formation of purple azo dye at sites of activity. The sections were then immersed in distilled water for several minutes and finally mounted on glass slides with glychrogel.

Esterases: Esterase activity was demonstrated using naphthyl AS acetate as substrate. Eight-tenths of a millimeter of a 1 per cent stock solution (in 1:1 acetone and propylene glycol) was mixed with 12 ml. of propylene glycol. To this was added 37 ml. of distilled water, 5 ml. of 0.2 M phosphate buffer (pH 6.6) and 20 mg. of diazotized-o-amino azotoluene.† The preparation was vigorously shaken to ensure adequate mixing and filtered through Whatman No. 1 paper into a glass receptacle. The tissues were incubated at 37° C. for 45 to 60 minutes. After removal from the substrate solution, the sections were washed in distilled water and mounted. Sites of enzyme activity were visualized by a red-purple dye with a yellow "background" color present in nonreactive areas.

*Brentamine Fast Blue B; Imperial Chemical Industries Ltd., Manchester, England.
†Brentamine Garnet GBC; Imperial Chemical Industries Ltd., Manchester, England.

Beta-D-galactosidase: The substrate solution was prepared by dissolving 15 mg. of 6-bromo-2-naphthyl β-D-galactopyranoside in 10 ml. of methanol. Dissolution of the compound was aided by the addition of 15 ml. of boiling water. Upon cooling, 20 ml. of 0.1 M acetate buffer (pH 5.0) was added to the mixture. Next 5.8 grams of sodium chloride was placed in the flask and sufficient distilled water was added to yield a final volume of 100 ml. Maintained at room temperature this solution was stable for at least 2 months.

Fixed salivary gland tissues were incubated for 8 to 24 hours at 37° C. A separate receptacle was provided for each type of tissue and, immediately after the removal of a section for enzyme localization, an aliquot of the solution was tested for free 6-bromo-2-naphthol. Initially, the substrate solution contained no sodium chloride and solutions incubated for 8 hours had a light to moderate violet color when tested. With longer periods of incubation the color intensity was so great as to cause doubt to the validity of the localization. Addition of sodium chloride to the substrate, in an amount sufficient to produce a final 1 M concentration, substantially reduced diffusion of the enzyme into the substrate solution and permitted periods of incubation up to 24 hours. Average incubation time for salivary gland tissue was 16 hours.

Enzymatically liberated 6-bromo-2-naphthol was visualized by coupling with diazotized-o-dianisidine following the technic outlined for acid phosphatase.

RESULTS

Parotid Gland—

Acid phosphatase: Reaction in this gland, characterized by a homogenous red-violet stain throughout the cytoplasm of reactive cells, was generally limited to the acini and ducts. Intralobular and excretory duct cells were of equal reactivity. All duct types were somewhat more reactive than acinar cells. Color was discernible in the basal membrane of the acini, but because of the delicate nature of this structure it was not possible to compare the color with the reaction intensity of other positive structures. Connective tissue was for the most part negative. However, the tunica media of arterioles was lightly stained (Fig. 1).

Nonspecific esterases: Esterase activity in the parotid gland structural elements presented a completely different type of reaction from acid phosphatase. A conspicuous amount of red granules were dispersed throughout the cytoplasm of acinar and duct cells. The cytoplasmic background and basal membrane of the acinar cells were unstained. Duct epithelium was more heavily stained than the acinar cells; the granules being larger in size and greater in quantity so that the entire cytoplasm appeared reactive. Intralobular, interlobular, and excretory ducts had more or less the same color intensity (Fig. 2).

Beta-D-galactosidase: Galactosidase activity produced a stain similar to that seen for acid phosphatase, but lighter in intensity. The cytoplasm of acinar

Fig. 1.

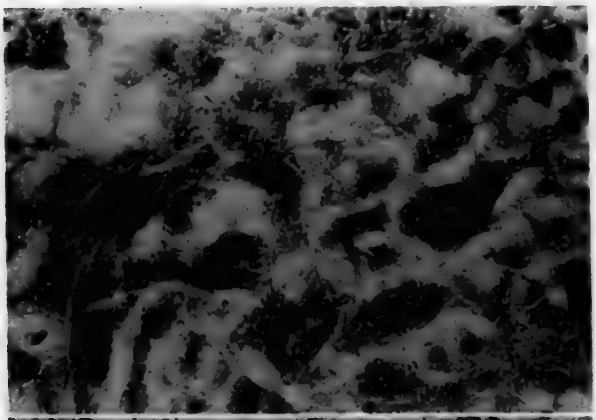
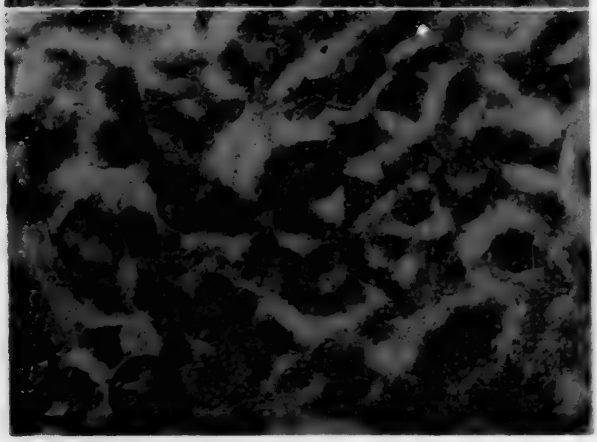


Fig. 2.

Fig. 3.



Figs. 1, 2, and 3.—Parotid Gland. (×120; reduced ½.)
Fig. 1.—Acid phosphatase: 6-benzoyl-2-naphthyl phosphate.
Fig. 2.—Total esterase: naphthyl AS acetate.
Fig. 3.—Galactosidase: 6-bromo-2-naphthyl β-D-galactopyranoside.

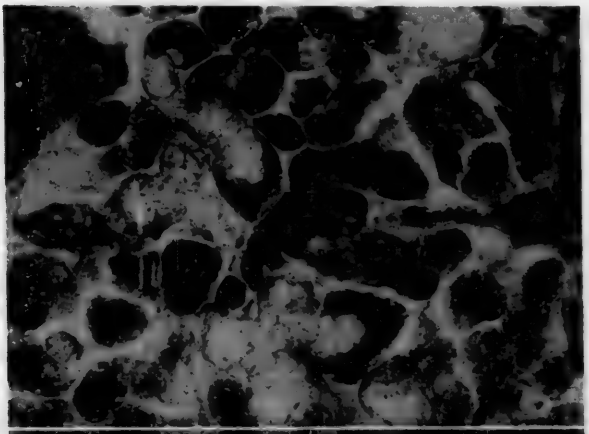


Fig. 4.

Fig. 5.

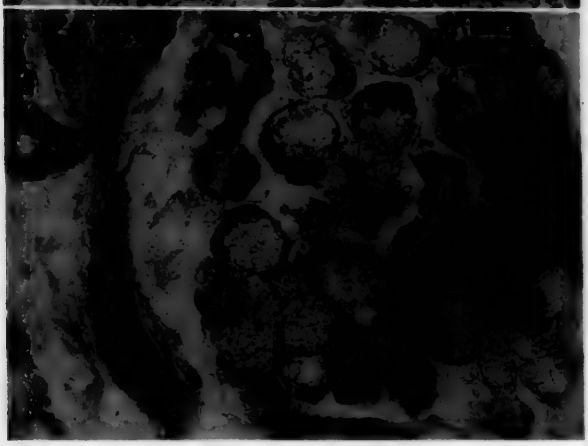


Fig. 6.

Figs. 4, 5, and 6.—Submaxillary Gland. (×120; reduced ⅓.)
Fig. 4.—Acid phosphatase: 6-benzoyl-2-naphthyl phosphate.
Fig. 5.—Total esterase: naphthyl AS acetate.
Fig. 6.—Galactosidase: 6-bromo-2-naphthyl β-D-galactopyranoside.

epithelium contained a homogenous light violet color. All duct types were stained with equal intensity, but exhibited less activity than the acinar components. As with acid phosphatase, the basal membrane of the acini, while not markedly colored, was clearly visible (Fig. 3).

Submaxillary Gland.—

Acid phosphatase: Serous acini and demilunes were heavily stained. Intralobular, interlobular, and excretory ducts were strongly positive, exhibiting the same degree of intensity between duct types; a color which was similar in magnitude to that noted in the acini. The basal membrane was quite dark especially in mixed (serous and mucous) acini. Mucous cells were considered to be negative although some small granules were occasionally seen within these cells (Fig. 4).

Nonspecific esterases: The reaction observed was quite similar to that noted with parotid gland tissue. Red granules were present in the serous acini which were otherwise not stained and did not present a visible basal membrane. Demilunes were also positive, but had a well-defined basal membrane which separated them from the nonreactive mucous acini. The cytoplasm of all ducts was intensely reactive and filled with red granules of various sizes (Fig. 5).

Beta-D-galactosidase: Serous acini presented a homogenous reaction which was moderate in intensity. The basal membrane, though lightly colored, was clearly visible. Demilunes were also positive and presented a marked contrast with their adjacent mucous cells which were negative. This division between mucous and serous components was enhanced by the marked reactivity of the basal membrane of the mucous cells. Ducts had the same intensity of color as acinar cells, and showed no gradation between duct types (Fig. 6).

All enzyme activity was thus confined to the cytoplasm and intimate structures of the serous components, acini and ducts. Nuclei were never observed to contain activity. Similarly, adipose tissue, blood vessels, and connective tissue were generally negative. Mucous cells exhibited activity only for β-D-galactosidase and then this was limited to the basal membrane of these cells.

Effects of Sex and Age.—

No difference was noted between male and female subjects. However, reaction intensity appeared to be related to subject age. Tissues from infants demonstrated a greater degree of activity than adult tissues. Components which were usually negative in adult glands, blood vessels, and connective tissue, were slightly reactive in infant tissues.

DISCUSSION

Two types of staining reaction were observed. One type was characterized by a diffuse over-all stain which usually covered the entire cytoplasm of the cells. The other type was a refractive granular stain which could be either dispersed throughout the entire cytoplasm or limited to a specific region. Acid

phosphatase and β-D-galactosidase exhibited both types of staining. The granules present were quite fine and evenly distributed throughout the cytoplasm of reactive cells. Nonspecific esterase activity in the acinar cells was predominantly of the granular type, while ducts showed both the granular and diffuse reaction.

Table I gives the location and reaction intensity of the enzyme activities noted in human parotid and submaxillary glands. Some differences in reactivity occurred between the two glands. Acid phosphatase activity in the submaxillary gland acini was slightly greater than that observed in the serous components of parotid tissue. However, all reactive sites in the submaxillary gland were of equal intensity in contrast to the parotid gland where the ducts were slightly more reactive than the acinar cells. Esterase activity of both glands was similar in magnitude and localization. Beta-D-galactosidase activity of the serous components in the submaxillary glands was greater than that noted in the parotid acini. Acinar and duct epithelium in submaxillary tissue stained with equal intensity, in contrast to the parotid tissue where the acini were more heavily colored than the duct cells. An interesting feature was the reactivity of the basal membrane of mucous acini, a structure which demonstrated neither acid phosphatase nor esterase activity.

TABLE I

	ACID PHOSPHATASE	NONSPECIFIC ESTERASES	β-D-GALACTOSIDASE
	Parotid Gland		
Acini	3	1	1-½
Ducts			
Intralobular	4	3	1
Interlobular	4	3	1
Excretory	4	3	1
Blood Vessels	½	½	½
Connective Tissue	½	½	½
Adipose Tissue	0	0	0
	Submaxillary Gland		
Serous Acini	4	1	2
Demilunes	4	1	2
Ducts			
Intralobular	4	3	2
Interlobular	4	3	2
Excretory	4	3	2
Mucous Cells	0	0	1
Blood Vessels	½	½	½
Connective Tissue	½	½	½
Adipose Tissue	0	0	0

Reactivity

Intense—4+ Weak—1-1½
Marked—3-3½ Occasional—½
Moderate—2-2½ Negative—0

These findings indicate that, except for minor differences, the enzyme and localization in the serous components and ducts of the parotid and submaxillary glands appear similar. Earlier observations[11] on the enzyme contents of the parotid and submaxillary secretions have shown that significant amounts of acid phosphatase and nonspecific esterases are present. The information obtained in

the present study, when evaluated in conjunction with past observations, suggests that the ducts as well as the acini actively contribute to the secretion produced by the parotid and submaxillary glands.

SUMMARY

The localization of three hydrolytic enzymes, acid phosphatase, nonspecific esterases, and β-D-galactosidase, in human parotid and submaxillary glands was determined. Enzyme reactions were generally confined to the cytoplasm of serous cells and ducts. Nuclei were never observed to contain activity. Adipose tissue and mucous cells were usually negative. Mucous cells exhibited only β-D-galactosidase activity and then this was limited to the basal membrane of these cells. No difference was observed in tissue obtained from male and female subjects. However, the reaction intensity appeared to be related to subject age. Specimens from infants demonstrated greater activity than adult specimens and components such as blood vessels and connective tissue, which were usually nonreactive in adult glands, were slightly reactive in infants.

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PERIPHERAL CAPILLARIES IN THE RODENT INCISOR PULP

D. ADAMS

Anatomy Department, Edinburgh University, Edinburgh, Scotland

INTRODUCTION

MANY textbooks describe a subodontoblastic capillary plexus in human teeth, and some mention that capillary loops may extend among the odontoblasts.[1, 2] In a description of the blood vessels in the teeth of dogs, rabbits, guinea pigs, and pigs, Lepkowski[3] described capillary loops reaching almost to the dentine in the rabbit, and to a lesser extent in the other animals. In a later paper,[4] he pictured capillaries lying adjacent to the dentine in human developing molars. Warwick James[5] called attention to the scarcity of literature on the details of the odontoblast-capillary relationship, and compared the latter to the arrangement of capillaries in bone. Saunders,[6, 7] using x-ray microscopy, described a "subdentinal" plexus in human teeth, and stated that "individual capillary loops appeared to lie within dental bays." Unfortunately it is impossible to get much information from his microradiographs on the relation of these capillaries to cells.

The nutritional importance of these capillaries is obvious in the production of dentine matrix and its subsequent calcification, and also in the calcification of enamel, which is thought by some authors to occur via the pulp (Jasswoin,[8] Lefkowitz, Bodecker, and Shapiro,[9, 10] Marsland[11]).

In this paper, the relationship of capillaries to odontoblasts in rodent incisors is described. These teeth, which have been the subject of many studies on calcification, eruption, endocrine problems, etc., exhibit persistent growth and all developmental stages throughout life, and their wide open bases favor the filling of capillaries with an injection mass.

MATERIALS AND METHODS

A number of rats, rabbits, hamsters, and mice were used, the technic being the simple injection of an India ink-sodium citrate mixture into one common carotid artery or the aorta. The mixture was prepared by centrifuging and vacuum-filtering the India ink to reduce the particle size below 10 μ, and adding an equal volume of 7.6 per cent sodium citrate. The perfusion was carried out with gentle pressure on a barrel syringe, immediately after killing the animal with chloroform or ether. It was found advantageous to wash out the red blood cells beforehand with a 3.8 per cent sodium citrate solution.

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Filling of the pulp vessels could be observed by using transmitted light and was indicated by an intense blackening of lips, tongue, and gums.

Celloidin sections, approximately 40-80 μ thick, were prepared after decalcification in 7 per cent nitric acid. This allowed the vessels to be followed farther through the tissue, and gave a better indication of the general arrangement than with paraffin sections, which were also prepared for comparison using injected and noninjected material.

The sections were stained with hematoxylin and eosin except in 1 case where a methylene blue-eosin stain (Mann[12]) was used after hematoxylin, with good results. The latter method was used when the India ink had failed to fill the capillaries and they were packed with red blood cells, which stained a distinctive reddish brown.

OBSERVATIONS

In the majority of the specimens, the capillaries were well filled, although here and there too vigorous pressure caused a localized extravasation of the injection material. Where the India ink did not penetrate, the red cells were usually packed into the capillaries and made them easily visible.

The arrangement in the different species was similar and the picture in the rat was typical.

The main vessels of the pulp in longitudinal section were seen to occupy the lingual or concave half of the pulp. The arteries could be distinguished as narrow straight vessels while the veins were wider and more irregular channels. From the arteries, long arterioles arose on the buccal aspect and coursed obliquely through the pulp before turning in between the odontoblasts (Fig. 1). On the lingual aspect the arterioles did not run such a long course and many of them ran in a recurrent direction toward the odontoblast layer.

Many of the arterioles of both kinds gave slender branches to the body of the pulp tissue and also anastomosed loosely with neighboring vessels on the internal surface of the odontoblasts. This subodontoblastic plexus was more marked in the rabbit and hamster, while it had a very loose character in the rat (Fig. 2). Terminal branches of the arterioles ran through the odontoblast layer, more or less parallel to the long axis of the cells, and ended by joining a very dense plexus of capillaries just internal to the predentine. This plexus was formed by a rich anastomosis of adjacent vessels. It was difficult to differentiate with certainty between the efferent and afferent channels except where they could be followed to a main trunk, but there appeared to be a larger number of venous pathways leaving the plexus. The plexus at the distal end of the odontoblasts will be referred to as the *predentinal plexus* in order to distinguish it from the looser subodontoblastic plexus.

In longitudinal sections the predentinal plexus appeared, under low power, as an almost unbroken black line just internal to the predentine (Fig. 1). High-power views of the plexus showed that there was always an extension of the cytoplasm of the odontoblasts between the capillaries and the predentine. Tomes's processes which adjoin a capillary appeared to bend round the vessel

Fig. 1.

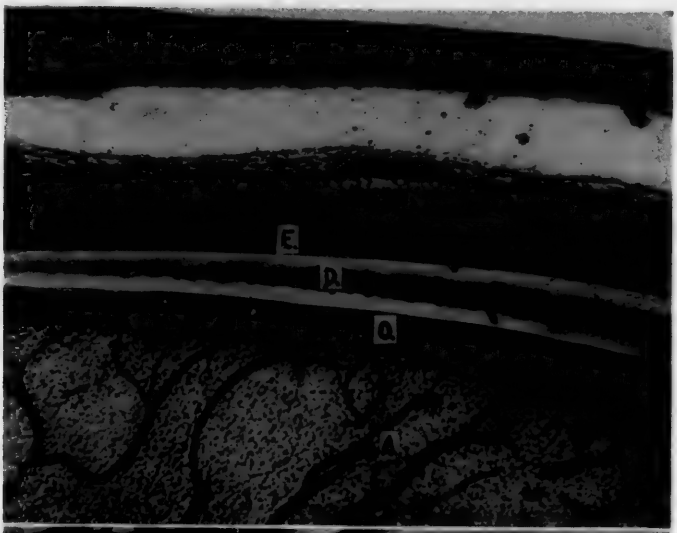
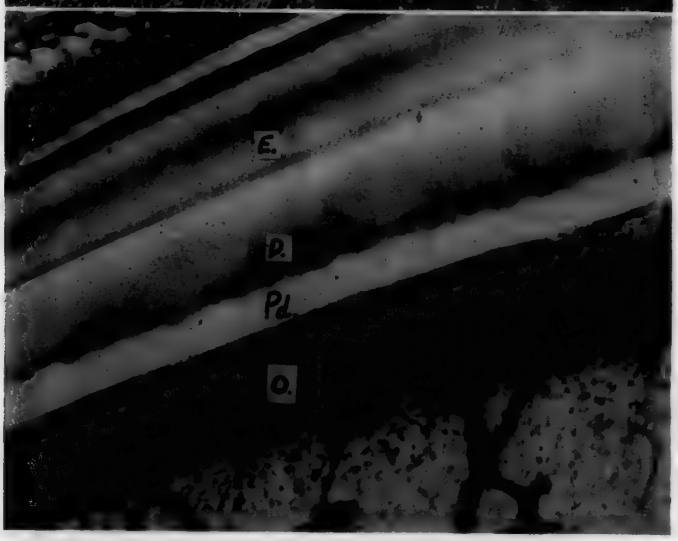


Fig. 2.



All the photomicrographs, except for Figs. 3, 5, and 7, are of longitudinal sections, approximately 60 μ thick, of rat incisors after the animal had been perfused with India ink mixture.

Abbreviations used

A = artery and arteriole.
C = cementum.
Cap = capillary.
D = dentine.
E = enamel matrix or enamel space.
O = odontoblasts.
P = pulp.
Pd = predentine.
P.M. = periodontal membrane.
V = vein and venule.

Fig. 1.—The large blood vessels can be seen running anteriorly through the pulp tissue, terminating in the odontoblast layer. (×60.)

Fig. 2.—A higher power view of part of Fig. 1. The terminal *predentinal plexus* is shown lying adjacent to the predentine. (×200.)

as was seen best in thin paraffin sections of noninjected material (Fig. 3). In these sections the capillaries were seen in cross section, as endothelial-lined tubes, containing red blood cells in some places. Sections tangential to the predentine-pulp junction showed the predentine plexus to advantage, and its density could be appreciated (Fig. 4).

Fig. 3.

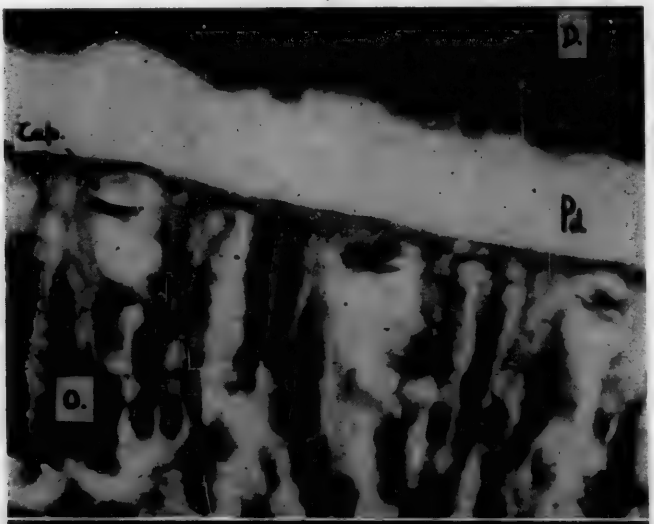


Fig. 4.

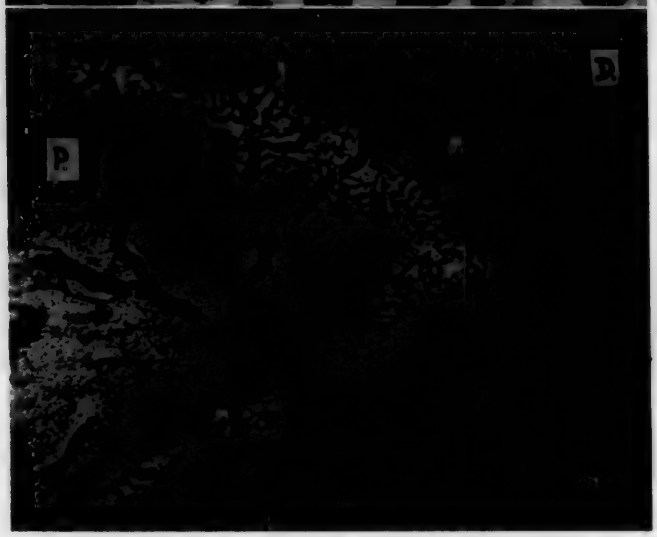


Fig. 3.—Thin section (10 μ) of rat incisor, not perfused. The Tomes's processes bend round the capillaries to reach the predentine. There appears to be an extension of odontoblast cytoplasm intervening between the capillary and the predentine. (×400.)

Fig. 4.—This section cuts the dentine-pulp surface tangentially. The density and complexity of the capillary bed is very marked. (×80.)

In the rabbit, the plexus was composed of finer vessels while the subodontoblastic capillaries on the whole were wider (Fig. 5). The hamster presented an arrangement indistinguishable from the rat.

Transverse sections of the incisors were used to compare directly the blood vessel-odontoblast relationship on the buccal aspect (enamel-covered) with that on the cement-covered part of the tooth. Apart from the difference in length of the odontoblasts, which were shorter under the cement-covered dentine, no obvious difference was seen in the two regions. The capillaries were again arranged under the predentine (Figs. 6 and 7) on the lingual aspect of the tooth, but the subodontoblastic plexus was composed of vessels of a larger caliber than those under the enamel-covered part of the tooth.

Fig. 5.—Rabbit incisor. The predentinal plexus is made up of fine capillaries, while the subodontoblastic vessels are larger than in the rat. (×200.)

From the base of the incisor, the development of the two systems of capillaries could be followed (Figs. 8 and 9). At the base, capillaries lay relatively far from the differentiating odontoblasts. Where the odontoblasts formed a uniform regular layer, one cell thick, the capillaries were found nearer and nearer the nuclear ends of the cell. As the dentine matrix became calcified, as judged by the staining reaction with hematoxylin, the odontoblast layer lost its regularity and became a layer of pseudostratified crowded cells. The capillaries at this level passed between the odontoblasts, and as the dentine layer became thicker they came to lie closer to the predentine, until they reached the position described above (see Fig. 2). Here they anastomosed freely with other loops and formed a rich network among the distal ends of the odontoblasts.

This penetration of the odontoblast layer would seem to be an outgrowth of new capillaries, since the original subodontoblastic vessels appeared to remain at the pulpal ends of the cells. At the incisive end of the pulp, where the

Fig. 6.

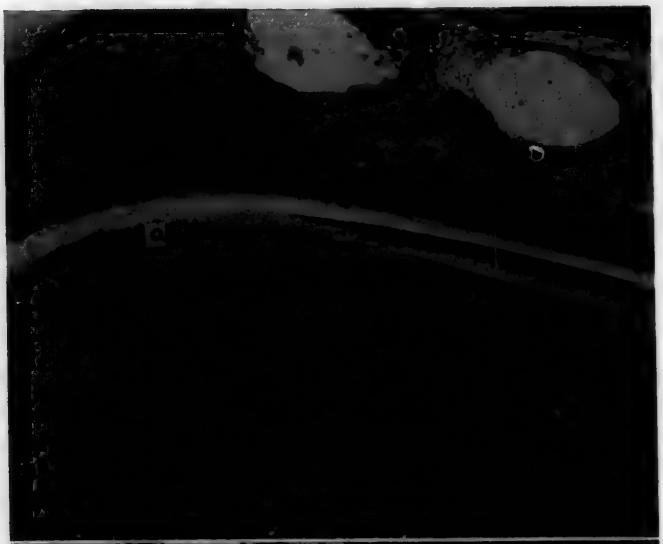


Fig. 7.

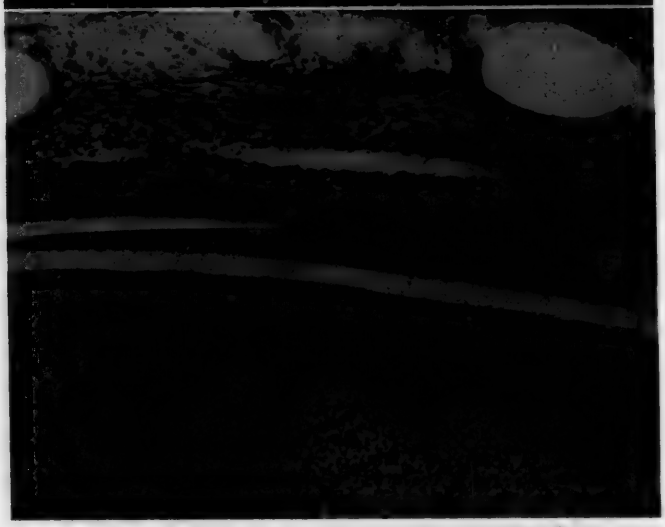


Fig. 6.—Under the cement-covered dentine, the capillaries bear the same relation to the predentine and odontoblasts as under the enamel-covered dentine. The veins and arteries supplying these capillaries often run in a recurrent direction before joining the main vessels. (×135.)

Fig. 7.—Transverse section (40 μ thick) of rat incisor. A direct comparison of the position of the capillaries relative to the odontoblasts under cement-covered dentine with those under enamel-covered dentine can be made in this section. The odontoblasts themselves are shorter but there is no difference apparent in the arrangement of the capillaries. (Stained by Mann's method; ×40.)

Fig. 8.

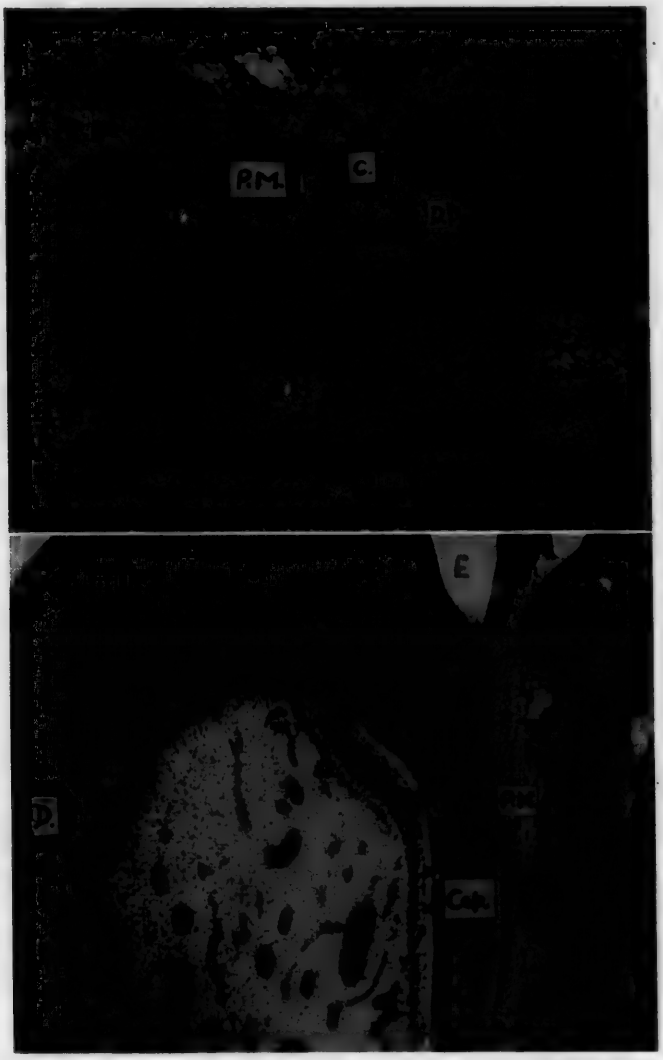


Fig. 9.

Fig. 8.—Developing end of the incisor. The cell-rich pulp has numerous capillaries but no vessels are directly related to odontoblasts at this level. (×135.)

Fig. 9.—Section to the right of Fig. 8, moving toward the incisive edge. Capillaries penetrate the odontoblasts and reach at the right the position shown in Fig. 2. The subodontoblastic plexus can also be seen in this illustration. (×135.)

odontoblasts were degenerating, the predentine plexus was still present, although reduced in number of vessels (Fig. 10); at the apex of the pulp the narrow cavity appeared to be filled with large blood vessels alone.

No interspecies differences in the mode of development were found, nor were any variations found between the formation of the predentine plexus on the buccal and lingual aspects of the pulp.

Venous channels, distinguished by their continuity with the wide vessels in the center of the pulp, were very numerous. The veins themselves were not found to become narrower toward the base of the pulp, as Saunders[6] found in human teeth, but remained large and thin walled.

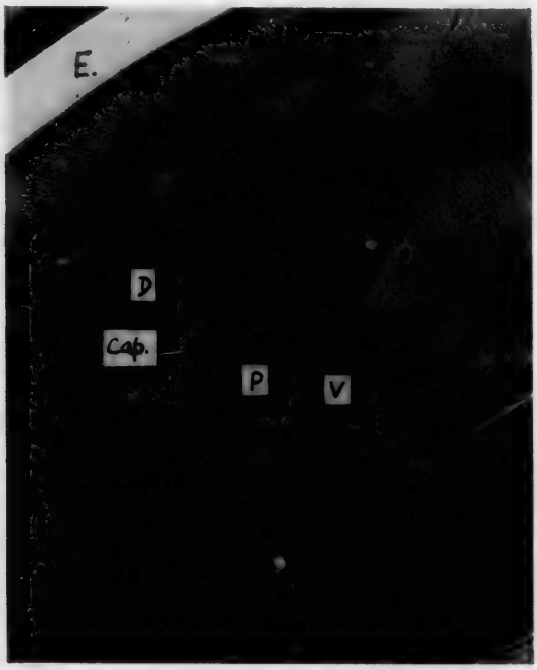


Fig. 10.—Section near the tip of the pulp. The odontoblasts are degenerating but capillaries can still be seen related to the predentine. (×40.)

DISCUSSION

The capillaries in the odontoblastic region of the rodent incisor pulp have approached the predentine to a point just short of producing a vasodentine. In none of the specimens examined were vascular inclusions in the dentine found. Saunders[6] has described an arrangement similar to this in adult human teeth using microradiographs, although histologic confirmation would be valuable. Apart from this, capillary networks so close to the predentine appear to be associated with an active laying down of dentine. Fish[13] stated that, under experimental cavities in the dentine of monkeys, the capillaries invade the

odontoblast layer and "these vessels come to lie between the injured dentine and the odontoblasts." After a few weeks, however, these capillaries recede and the surviving odontoblasts form a reduced odontoblastic layer and lay down secondary dentine. But it is possible that the secondary dentine was laid down while the capillaries were close to the dentine. The arrangement in the rodent incisor would appear, then, to resemble the reaction under injured dentine in the monkey or that found in developing teeth of a number of animals,[3, 5] although the presence of a dual set of capillary networks, i.e., subodontoblastic and predentinal, is not mentioned by these workers. It may be that in teeth where dentine is being actively laid down, e.g., the rodent incisor or developing teeth of limited growth, it is necessary for materials to be brought as close to the site of calcification as possible, where they will be acted upon by substances produced by the odontoblasts or other pulp cells. It is interesting in this connection to note that Hoffman and Schour[14] found in rodents, kittens, and puppies a production rate of dentine of 16 μ per day, which was constant for the three species.

There is no noteworthy difference in the proximity of the capillaries to the predentine under the cement and that under the enamel. Thus, there is no evidence of any special anatomic arrangement of capillaries to provide for the calcification of enamel through the dentine, but the possibility of this is not excluded.

In a recent paper, Glasstone[15] has shown that the uncalcified dentine of normal rodent incisor teeth would calcify in vitro, even when the odontoblasts had been removed. This required a suitable medium in which the concentrations of calcium and phosphorus were comparable to those in the blood. No alkaline phosphatase was found in the formed matrix, but was present in the cells when the matrix was being laid down. She concluded that alkaline phosphatase is not necessary for calcification, and her experiments support the view that, in rodents, the necessary factors for calcification are present in the dentine matrix, and that calcification does not depend on the presence of odontoblasts. The presence of a plexus of capillaries so close to the predentine, which would presumably allow of a high concentration of essential minerals in this zone, together with the persistence of blood vessels in regions where the odontoblasts are degenerating, seems to be in keeping with this view.

Further support came from the fact that as the odontoblast layer became thicker, capillaries appeared to push their way through to become closer to the predentine, and so presumably allow a greater flow of tissue fluid to bathe the matrix.

The slight variations in the predentinal and subodontoblastic plexuses among the rodents examined were not considered significant.

SUMMARY

1. The capillary systems of the odontoblast layer of the incisors were examined in the rat, rabbit, hamster, and mouse. The technic used was that of India ink perfusion, and the examination of injected and noninjected material.

2. There existed a very close meshwork of capillaries adjacent to the predentine and lying among the distal ends of the odontoblasts. A looser network was found in the subodontoblastic region. Connections between these two were formed by vessels which mostly ran parallel to the long axes of the odontoblasts.

3. The development and fate of this plexus was traced from the formative to the incisal end of the tooth.

4. It is suggested that this arrangement may be related to rapid dentine production and that it is in keeping with the concept of a noncellular mechanism for the calcification of the dentine matrix.

I wish to thank Professor Schlapp of Physiology Department, University of Manchester, for some of the material, Professor Romanes and Dr. C. H. Kemball, Anatomy Department, Edinburgh University, for helpful criticism of the manuscript. I am indebted to Mr. H. Tully for assistance with the photomicrographs.

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STUDIES ON MUCOPOLYSACCHARASE ACTIVITY OF ORAL SPIROCHETES

EDWARD G. HAMPP,* STEPHAN E. MERGENHAGEN, AND ROBERT R. OMATA

National Institute of Dental Research, National Institutes of Health, U. S. Department of Health, Education and Welfare, Bethesda, Md.

IN VIEW of the presence of large numbers of spirochetes in cases of ulceromembranous gingivostomatitis, any demonstration of enzymes produced by these organisms which degrade the intercellular cementing substances of connective tissue may assume importance in evaluating the role of the spirochete in this disease. It has already been shown that mucopolysaccharases may indeed play a significant role in synergistic intracutaneous infections with certain oral bacteria, including the spirochete.[1, 2] Berger[3] has reported the elaboration by the small oral treponemes of extracellular enzymes which are active in degrading mucopolysaccharides of human synovial fluid. Dewar[4] has reported similar findings. The present report is the result of an extensive investigation of representative strains of all known types of oral spirochetes for mucopolysaccharase activity.

MATERIALS AND METHODS

The medium and cultural technics employed in this study have been described previously.[5, 6] Eight strains of the small oral treponeme, 7 strains of *Borrelia vincentii* and 1 strain of *Borrelia buccalis* were employed for purposes of this investigation.[5, 6, 7] As positive controls for hyaluronidase activity, culture filtrates of *Clostridium perfringens* (SR12), an oral strain of *Streptococcus pyogenes*, and a preparation of testicular hyaluronidase (Schering) were employed. Culture filtrates and cellular extracts of an oral strain of diphtheroid bacillus (D7) containing chondroitinase activity were also used as positive controls.[8]

Hyaluronidase activity was determined in turbidity reduction units (TRU).[9] Chondroitinase was assayed in a similar manner.[8] Potassium hyaluronate, prepared from human umbilical cord, and chondroitin sulfate, obtained from Nutritional Biochemicals Corporation, were employed as substrates. Further, human synovial fluid was employed as a substrate in the ACRA test[3] for the detection of hyaluronidase activity in cell-free filtrates of representative cultures of the small oral treponeme (FM), *B. vincentii* (N9) and *B. buccalis* (GT2).

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*Senior Research Associate, American Dental Association, National Institute of Dental Research, National Institutes of Health, Bethesda 14, Md.

In addition to testing spirochetal culture supernatants or cell-free filtrates at 3 and 7 days after inoculation for enzymatic activity, 1.75 grams dry weight of a small oral treponeme (FM), obtained by harvesting 13 L. of culture after 7 days' growth, was resuspended in 50 ml. 0.1 M acetate-NaCl buffer of pH 6.0 and placed in a 10 kilocycle Raytheon sonic oscillator to disrupt the cells. Also, 2.0 grams dry weight of the diphtheroid bacillus (D7), obtained by harvesting 5 L. of culture after 72 hours' growth, was treated in the same manner. Cellular debris was removed by high-speed centrifugation and assays were carried out on the crude supernatants to determine the presence of intracellular enzymes.

RESULTS AND DISCUSSION

A number of procedures has been used for the assay of mucopolysaccharases with substrates from a variety of sources; however, the turbidity reduction method has found most use as a highly sensitive and simplified procedure for the quantitative determination of enzymatic activity. The use of known hyaluronidase and chondroitinase-producing strains of bacteria demonstrated that the method of assay was sensitive enough to detect 0.1 turbidity reduction unit (TRU) of enzyme activity in cellular extracts as well as cell-free culture filtrates.

None of the spirochetes tested gave any evidence of activity against hyaluronic acid, chondroitin sulfuric acid, or human synovial fluid (Table I). Tests for the adaptive synthesis of hyaluronidase by spirochetes were carried out on a number of strains by the addition of varying amounts of hyaluronic acid to the culture medium employed. In no case could any hyaluronidase activity be found.

TABLE I

MUCOPOLYSACCHARASE ACTIVITY OF THE ORAL SPIROCHETES AND CONTROL MICROORGANISMS

	HYALURONIDASE		CHONDROITINASE		
	EXTRACELLULAR (TRU/ML.)	INTRACELLULAR† (TRU/ML.)	EXTRACELLULAR (TRU/ML.)	INTRACELLULAR† (TRU/ML.)	ACRA*
Small treponemes					
FM	0	0	0	0	–
NF, N39, JH, MRB, JB, NG, and N36	0		0		
Borrelia vincentii					
N9	0		0		–
N37, CON, N19, NP, SCN, and EH	0		0		
Borrelia buccalis—GT2	0		0		–
Diphtheroid—D7	64	16	24	4	
Clostridium perfringens—SR12	5				
Streptococcus pyogenes	15				
Testicular hyaluronidase					
15 TRU					+
150 TRU					+

+ = dissolution of mucin clot; – = no detectable dissolution of mucin clot.
*Cell-free culture filtrates tested.
†Soluble extracts of sonic oscillated cells.

These results are in contrast to those of Berger[3] who reported a weak hyaluronidase activity as evidenced by the action of culture supernatants of small treponemes on synovial fluid. The reason for this discrepancy is not clear; however, in the present study, in addition to synovial fluid, purified substrates were used and, furthermore, adequate controls with known strains of mucopolysaccharase-producing microorganisms were employed.

Evidence has been accumulated which indicates that the spirochetes possess pathogenic significance in human oral infections. Hampp[10] has shown that spirochetes were present in 21 out of 28 specimens obtained for cultural purposes from infected dental pulps unexposed to the oral secretions. Further, it has been observed, in biopsy material obtained from acute cases of ulcerative gingivostomatitis, that the oral spirochetes possess invasive potentialities since they were routinely found in the subepithelial connective tissue. (Hampp, E. G., unpublished data.)

Recent studies have shown that oral spirochetes can produce persistent abscesses in the skin of rabbits.[1] The fact that such lesions remain localized substantiates the conclusion that these microorganisms fail to produce spreading factors (i.e., mucopolysaccharases) in vivo as well as in vitro. If, however, a mucopolysaccharase-containing bacterial filtrate is injected into the periphery of the spirochetal lesion, there is a significant extension of the lesion in the following 24 hours. This result indicates that enzymes, produced by other oral bacteria in association with the spirochete, could give rise to a more intense infection, as is seen in certain human infections of the oral cavity where spirochetes predominate. Hoffman[11] has also postulated such a mechanism in relation to the pathogenesis of Vincent's infection.

SUMMARY

Sixteen representative strains of oral spirochetes (small oral treponemes, *Borrelia vincentii,* and *Borrelia buccalis*) were studied for the elaboration of mucopolysaccharase activity. In addition, an oral *Streptococcus pyogenes* and an oral diphtheroid bacillus were tested and served as controls. None of the spirochetes were found to produce enzymatic activity against hyaluronic acid, chondroitin sulfuric acid, or human synovial fluid. Extracellular hyaluronidase was detected in cultures of the streptococcus whereas the diphtheroid bacillus produced both extracellular and intracellular hyaluronidase and chondroitinase. These results, from in vitro experiments, indicate that the pathogenicity of the oral spirochetes does not depend upon their elaboration of mucopolysaccharases.

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SURVIVAL OF MOUSE TOOTH GERMS AFTER FREEZING IN LIQUID NITROGEN AS DEMONSTRATED BY DIFFERENTIATION OF ISOLOGOUS TRANSPLANTS

W. YOSHIOKA* AND F. GONZALES

Department of Anatomy, The University of Texas Dental Branch, Houston, Texas

THIS is a report of experiments designed to determine whether tooth germs will survive freezing to extremely low temperatures. The results are of value to those interested in transplantation because they indicate the possibility of preserving viable tooth germs indefinitely in the frozen state.

The literature on survival and preservation of frozen tissues has been reviewed.[1-4] One theory of death by freezing is that ice crystals are directly responsible for injury by expansion[1] or mechanical disturbance.[5] Another theory is that high salt concentrations resulting from precipitation of pure ice cause, among other chemical disturbances, dissolution of lipoproteins, which may lead to death.[6, 7, 8]

Before the discovery of the protective action of glycerol and related compounds on frozen tissue,[9-12] the emphasis was on ultrarapid freezing and thawing.[1] The importance of rapid freezing and thawing, when no protective treatment is used, has been recently re-emphasized.[4, 13, 14, 15] This factor becomes less important when tissue is pretreated in glycerol or ethylene glycol.

The fact that glycerol tends not to crystallize[15] and that it lowers considerably the speed of crystallization[16, 17] may help explain its protective action, but the theoretical background in this field remains poorly developed. As a result, optimal conditions for viable preservation in the frozen state must be determined empirically for each tissue.

Some of the criteria for viability of frozen-thawed tissues which have been used recently are muscle contractions,[18, 19] cell migration in tissue culture, [13, 16, 19, 20, 21] morphogenesis in tissue culture,[22] histogenesis in chorio-allantoic grafts,[23] and hemolysis of red blood cells.[6, 24]

In the experiments reported here, histogenesis of isologous transplants was used as a criterion of survival. By use of this test it was demonstrated that tooth germs of mice may survive temperatures down to –195° C. A marked protective action against freezing injury of mouse tooth germs by pretreatment in glycerol was also evident.

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*Present address: Osaka University Dental School, Osaka, Japan.

MATERIALS AND METHODS

Glycerol Treatment and Freezing.—Fetal C3H mice were removed from the mother and placed in a sterile Petri dish containing Tyrode's solution. Then, with the aid of a dissection microscope and fine jeweler's forceps, upper and lower first molars were carefully removed and placed in Tyrode's solution in another dish.

Harvested tooth germs were subjected to either of 2 experimental treatments. Some were frozen rapidly in liquid nitrogen on thin aluminum foil slips without having been exposed to glycerol. Others were soaked for 10 minutes in a 30 per cent solution of glycerol (Tyrode's) prior to the freezing. Frozen tooth germs in both groups were thawed rapidly by immersion in Tyrode's solution at 37° C.

It was necessary to provide two types of controls. Some tooth germs were soaked in 30 per cent glycerol (Tyrode's) for 10 minutes and then put back in ordinary Tyrode's without freezing. Others were neither treated in glycerol nor frozen.

Transplantation.—After the procedures just described, the tooth germs were either put into the anterior chamber of the mouse eye according to the method described by Fleming,[25] or they were transplanted subcutaneously to the axilla via a dorsal incision. Transplants were harvested 30 to 44 days later.

Histologic Preparation.—Ten per cent formalin was used as fixative. When necessary, the tissues were decalcified in 4 per cent hydrochloric acid. Paraffin sections were cut and stained with hematoxylin and eosin.

RESULTS

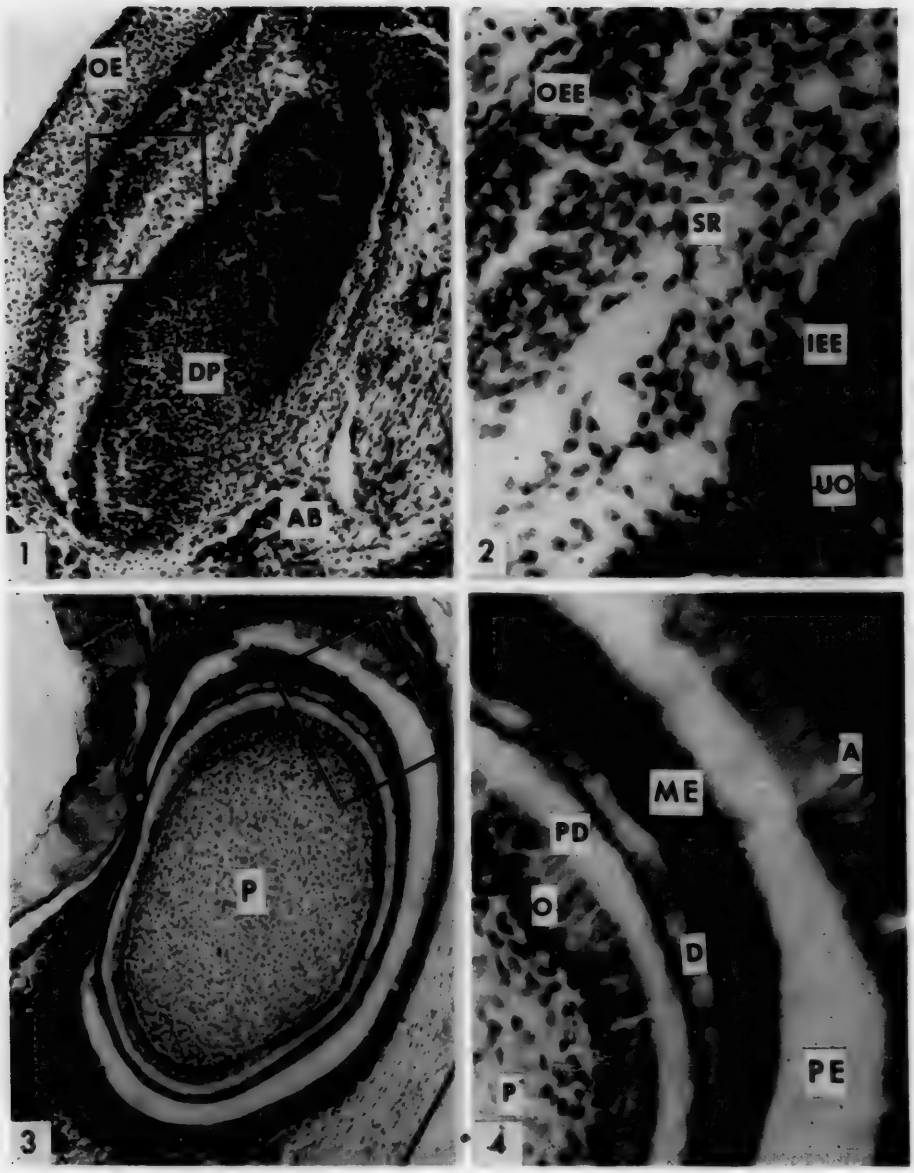
When the tooth germs were transplanted they contained neither enamel nor dentin. Odontoblasts had not yet differentiated. In the inner enamel epithelium, the nuclei had not migrated (Figs. 1 and 2). Our criteria for judging development consisted of the unfolding of these features which were absent in the original transplants. Only those transplants showing development were scored as positive takes.

Some untreated control transplants were recovered after 14 days in the host. These transplants had developed as shown in Figs. 3 and 4. Odontoblasts and ameloblasts were well differentiated. Moreover, dentin and enamel were present.

Eye Chamber Transplants.—A total of 77 transplants were made into the anterior eye chambers. This total consisted of the following groups.

Eighteen tooth germs which had been frozen in liquid nitrogen and thawed rapidly without glycerol pretreatment were transplanted to anterior eye chambers of isologous hosts. No trace of the transplants could be found at the end of 30 days.

Of those tooth germs which had been pretreated in 30 per cent glycerol and then frozen in liquid nitrogen, 24 were transplanted to the eye. Seven



Figs. 1 and 2 are photomicrographs of sections of excised, nontransplanted tooth germs. Figs. 3 and 4 are transplanted to the anterior chamber of the mouse eye.

Fig. 1.—Maxillary first molar from a 17-day mouse fetus. Not transplanted. The tooth germ contained neither dentin nor enamel. Odontoblasts had not yet differentiated. In the inner enamel epithelium, the nuclei had not migrated. Stellate reticulum was still present. (Mag. ×92.)

Fig. 2.—Higher magnification of area in rectangle in Fig. 1. (Mag. ×368.)

Fig. 3.—Glycerol pretreated but unfrozen mandibular first molar from a 17-day mouse fetus transplanted to the anterior chamber of the eye of an adult, male mouse and removed 14 days later. (Mag. ×92.)

Fig. 4.—Higher magnification of area in rectangle in Fig. 3. (Mag. ×368.)

Legend: AB—alveolar bone; *DP*—dental papilla; *IEE*—inner enamel epithelium; *OEE*—outer enamel epithelium; *UO*—undifferentiated odontoblast; *OE*—oral epithelium; *SR*—stellate reticulum; *O*—odontoblasts; *A*—ameloblasts; *E*—enamel; *D*—dentin; *PD*—predentin; *P*—pulp; *ME*—enamel matrix; *PE*—enamel space.

of these were successful (Figs. 5 and 6). In some of the transplants no tooth germs developed in spite of the fact that there was persistence and growth of the transplanted tissue. Most cells apparently intermixed with donor cells to such an extent that the tooth germs lost their morphological characteristics. Thus, the proportion of successful takes, estimated by gross observation, was higher than that revealed by histologic observation. However, in the numerical tabulation (Table I), only those tooth germs showing histologic differentiation were listed as successful.

TABLE I
SUMMARY OF RESULTS

	NUMBER OF TRANSPLANTS	NUMBER SHOWING TOOTH DEVELOPMENT	PER CENT
Eye Chamber			
Frozen without glycerol	18	0	0
Frozen with glycerol	24	8	33
Glycerol controls	16	7	44
Untreated controls	19	16	84
Axilla			
Frozen without glycerol	53	2	4
Frozen with glycerol	63	36	57
Glycerol controls	48	34	71
Untreated controls	55	48	87

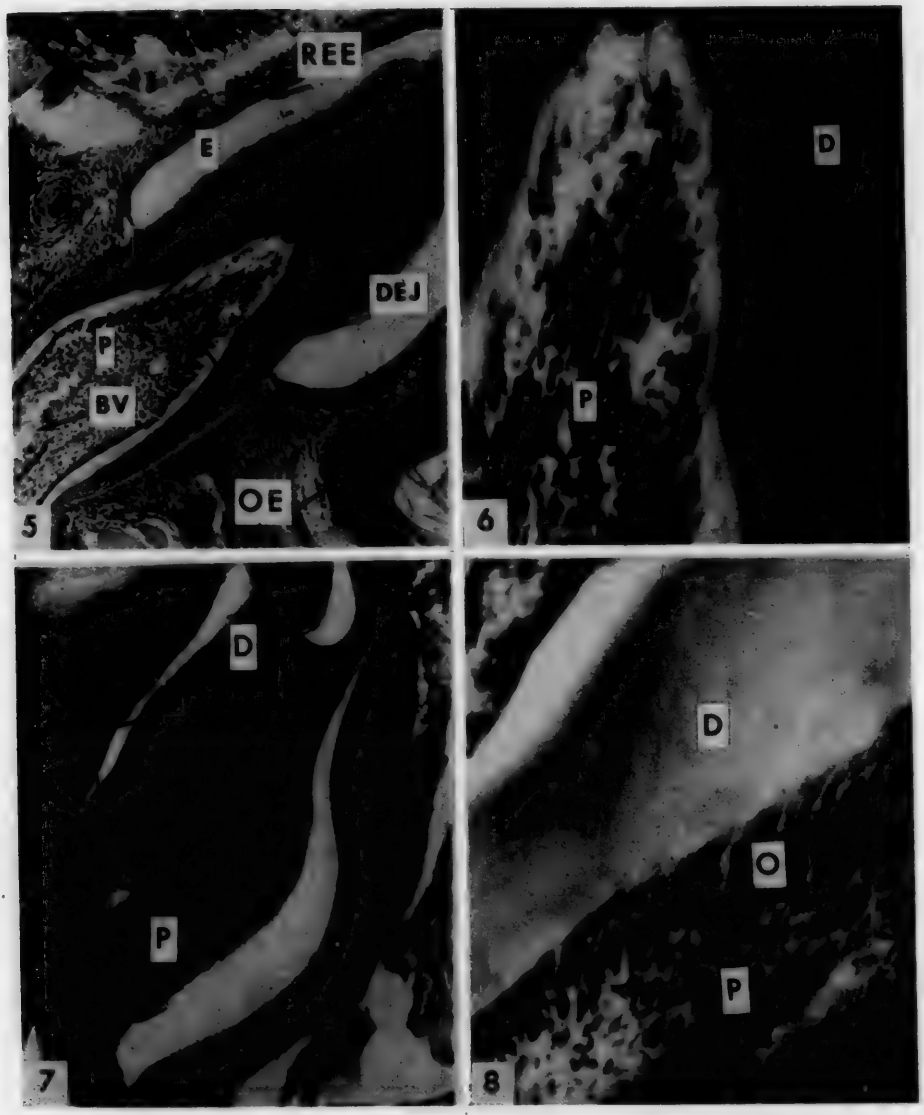
Of 16 glycerol-pretreated but unfrozen tooth germs transplanted to the eye, 7 were successful (Figs. 7 and 8). Under the conditions of this experiment, growth was not inhibited, so that in 35 days the entire anterior chamber was filled with tissue.

Nineteen tooth germs which had been neither frozen nor pretreated in glycerol were transplanted to the eye. Sixteen developed (Figs. 9 and 10). Some untreated control transplants continued to grow until they erupted from the anterior chamber.

Axilla Transplants.—Two hundred and nineteen tooth germs were transplanted subcutaneously in the axillae of mice of the same strain. The same experimental procedures were carried out on tooth germs that were transplanted into the axilla as on those that were put into the eye. The proportion of successful takes of untreated control tooth germs was the same in both groups. However, tooth germs that had been treated in glycerol and/or frozen were more often successful when put in the axilla than when put in the eye (see Table I for a summary of results).

Two of 53 tooth germs which had been frozen in liquid nitrogen without any protective pretreatment showed surprisingly normal development (Figs. 11 and 12).

Sixty-three tooth germs treated in glycerol and frozen in liquid nitrogen were transplanted to axillae. Thirty-six of this group showed good development of dentin, enamel and, in most cases, cementum (Figs. 13 and 14). Other transplants in this group contained alveolar bone and/or epithelium but not



Figs. 5 through 8 are photomicrographs of sections of transplants to the anterior chamber of the mouse eye.

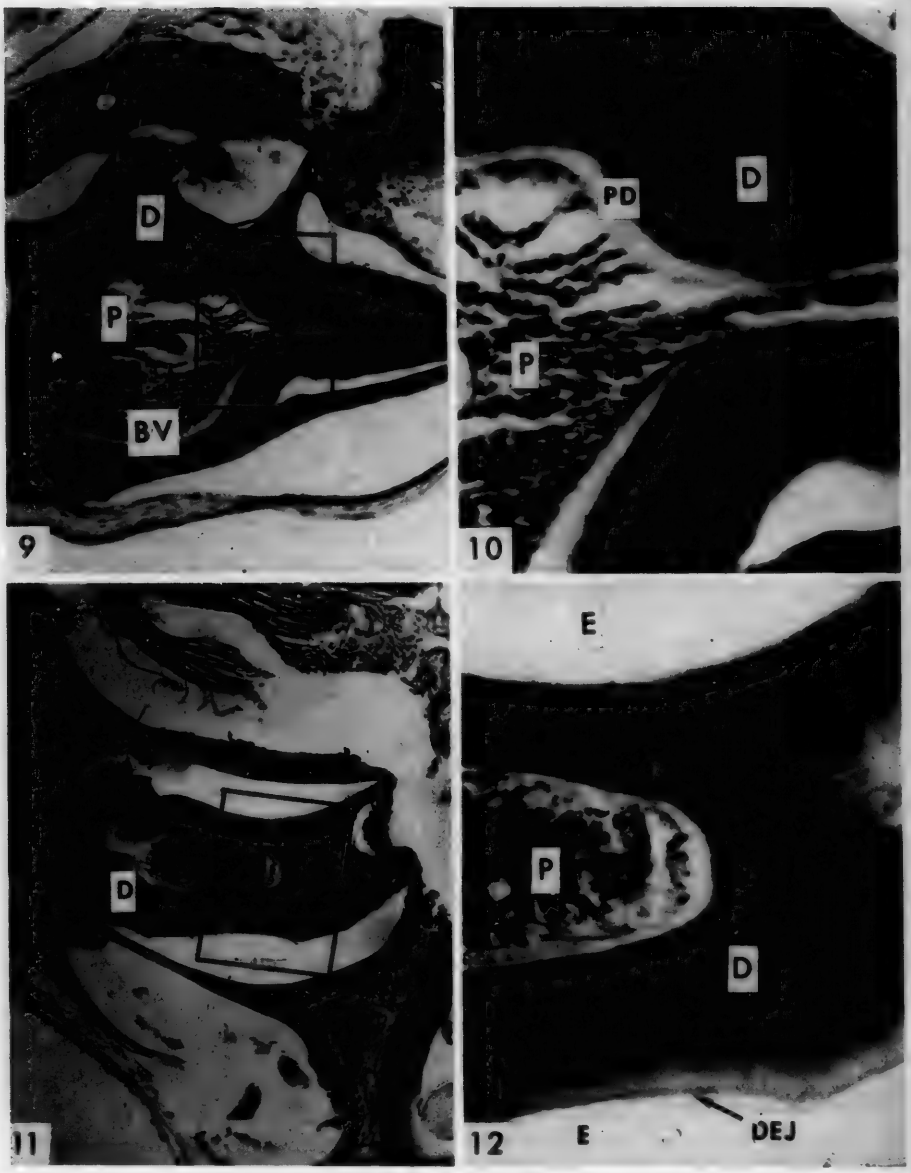
Fig. 5.—Photomicrograph of a section from a fetal mouse first molar. This tooth germ was pretreated in glycerol and frozen in liquid nitrogen. After thawing, the tooth germ was transplanted to the anterior eye chamber of an adult male mouse and removed 43 days later. (Mag. ×92.)

Fig. 6.—Higher magnification of area within rectangle in Fig. 5. (Mag. ×368.)

Fig. 7.—Photomicrograph of a section from a fetal first molar. This tooth germ was treated as in the one represented in Fig. 5 except that it was not frozen. (Mag. ×92.)

Fig. 8.—Higher magnification of area within rectangle in Fig. 7. (Mag. ×368.)

Legend: *D*—dentin; *E*—enamel; *DEJ*—dentinoenamel junction; *P*—pulp; *BV*—blood vessel; *O*—odontoblasts; *REE*—reduced enamel epithelium; *OE*—oral epithelium.



Figs. 9 and 10 are photomicrographs of sections of tooth germs transplanted to the anterior chamber of the mouse eye. Figs. 11 and 12 are photomicrographs of sections of tooth germs transplanted to the axilla of the mouse.

Fig. 9.—Glycerol-treated but unfrozen mandibular first molar from a 19-day mouse fetus transplanted to the anterior chamber of the eye of an adult, male mouse and removed 34 days later. (Mag. ×92.)

Fig. 10.—Higher magnification of area within rectangle in Fig. 9. (Mag. ×368.)

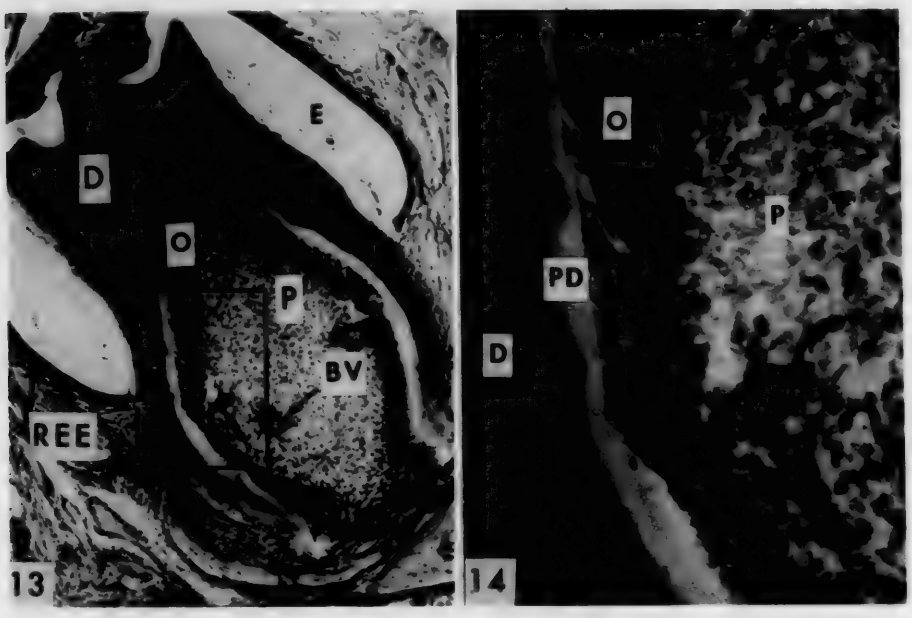
Fig. 11.—Maxillary first molar from 17-day fetus. Frozen without pretreatment in glycerol, transplanted to the axilla of an adult mouse and removed 35 days later. (Mag. ×92.)

Fig. 12.—Higher magnification of area within rectangle in Fig. 11. (Mag. ×368.)

Legend: *D*—dentin; *E*—enamel; *P*—pulp; *PD*—predentin; *BV*—blood vessels; *DEJ*—dentinoenamel junction.

tooth structures. Cyst-like structures containing heavy layers of keratin or, in some instances, hair were often found. It is interesting to note that when hair was present it was never pigmented even though C3H mice normally have pigmented hair.

Thirty-four out of 48 axilla transplants treated in glycerol, but not frozen, developed. The macro- and microscopic findings were essentially the same as those in other groups (Figs. 15 and 16). This was true also for the untreated controls transplanted to the axilla (Figs. 17 and 18). Forty-eight out of 55 of the latter showed tooth development.



Figs. 13 and 14 are photomicrographs of sections of tooth germs transplanted to the axilla of the mouse.

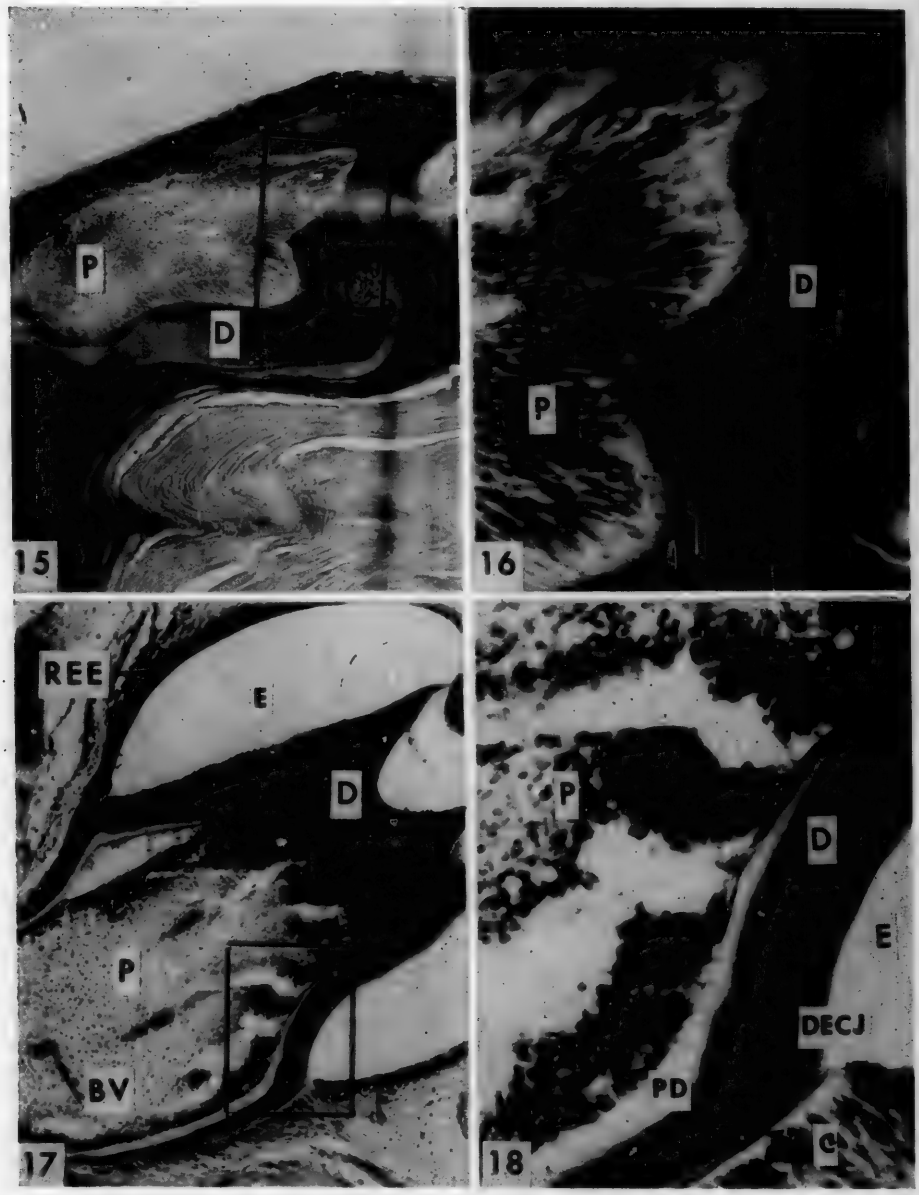
Fig. 13.—Photomicrograph of section from mandibular first molar tooth germ frozen in liquid nitrogen after glycerol pretreatment. The transplant was made to the axilla and recovered 33 days later. (Mag. ×92.)

Fig. 14.—Higher magnification of area within rectangle in Fig. 13. (Mag. ×368.)

Legend: *D*—dentin; *E*—enamel; *PD*—predentin; *O*—odontoblasts; *BV*—blood vessel; *REE*—reduced enamel epithelium; *P*—pulp.

DISCUSSION

The fundamental fact established in the preceding experiments is that tooth germs can be frozen to extremely low temperatures and still remain viable. The observation that glycerol-pretreated frozen tooth germs yield a lower percentage of takes than those treated in glycerol but not frozen indicates that there was considerable cell damage as a result of the freezing. However, glycerol treatment in itself contributes to the injury, as evidenced by a lower proportion of takes in unfrozen but glycerol-treated tooth germs



Figs. 15 through 18 are photomicrographs of sections of tooth germs transplanted to the axilla of the mouse.

Fig. 15.—Maxillary first molar tooth germ treated in glycerol but not frozen. Transplant was made to the axilla and recovered 35 days later. (Mag. ×92.)

Fig. 16.—Higher magnification of area within rectangle in Fig. 15. (Mag. ×368.)

Fig. 17.—Untreated control mandibular first molar from a 17-day mouse fetus transplanted to the axilla of an adult mouse and removed 35 days later. (Mag. ×92.)

Fig. 18.—Higher magnification of area within rectangle in Fig. 17. (Mag. ×368.)

Legend: *D*—dentin; *E*—enamel; *PH*—predentin; *O*—odontoblasts; *BV*—blood vessel; *REE*—reduced enamel epithelium; *P*—pulp; *DECJ*—cementoenamel junction.

compared with untreated controls. Thus, for optimum survival, it is necessary to find a compromise concentration of glycerol which, in itself, causes minimum damage but still protects against freezing injury.

The mechanism of injury resulting from treatment with glycerol alone is not completely understood. However, there is some evidence that it has to do with tonicity of the various media used. If a suspension of embryonic chick heart cells is exposed to a solution of glycerol in Tyrode's solution and then centrifuged and resuspended in pure Tyrode's, the cells die. If instead the cell suspension in glycerol solution is dialyzed against pure Tyrode's, a large proportion of the cells survive.[23] A possible interpretation of this observation follows. Glycerol diffuses through the cell membrane slowly compared to the other solutes making up the medium. Thus, when glycerol-impregnated cells are suddenly confronted with pure Tyrode's the glycerol remains long enough within the cell to make Tyrode's hypotonic. Dialysis acts to buffer the osmotic shock by a gradual removal of the glycerol from the medium.

Our results show that glycerol enhances greatly the resistance of tooth germs to cold injury. However, as mentioned above, 2 out of 53 tooth germs, frozen *without* any protective pretreatment, developed after having been transplanted to the axilla. There have been other reports of vertebrate cells surviving deep freezing without protective pretreatment.[15, 26] However, we did not expect enough unprotected cells to survive freezing in liquid nitrogen to yield such good examples of tooth development. Although the possibility remains that the tissues in these two tooth germs could have been supercooled at –195° C. for 10 minutes, it seems highly improbable. Rey[15] is of the opinion that unpretreated cells that survive freezing have an intrinsic resistance to freezing injury. It would be interesting if one could select and propagate such surviving cells by using tissue culture cloning procedures[27] and thereby develop cold-resistant strains of cells. A variety of chromosomal, biochemical, and biophysical methods could then be used to compare cold-resistant cells with ordinary cells.

Greene[28] found that the anterior chamber of the eye was ideally suited to receive transplants of certain embryonic tissue. Fleming[25] reported that anterior chamber transplantation yielded better results with tooth germs than did subcutaneous transplantation. Our results indicate that the axilla is a more favorable site for tooth germs than the anterior eye chamber. However, in our experiments, the eye chamber transplants were not entirely comparable with those in the axilla. Tooth germs destined to be transplanted to the eye were trimmed as completely as possible of surrounding tissue because of space restrictions, whereas those that were put in the axilla were incompletely dissected and were always transplanted along with some alveolar bone and oral epithelium. This may account for the lack of agreement between our results and those of Greene and Fleming. Another important fact in this connection is that it was only in the treated groups that any difference in proportion of survivors was evident between the eye chamber group and the axilla group.

Untreated controls in both groups yielded about the same proportion of unsuccessful grafts. This suggests that the axilla was more favorable as a site for tooth germs that had been injured in some way before the transplantation.

In certain grafting situations, for example, bone or artery grafts, the viability of the graft is not essential. In such cases the graft merely provides a scaffolding upon which cells from the host may migrate and revitalize.

That this could be the case in our experiments seems most unlikely for the following reasons: (1) the grafts were made to ectopic sites, (2) the structures which result, that is, dentin, cementum, among others, were not yet developed when the grafts were made, and (3) the gross form of the tooth developed mostly after the grafting and was very nearly normal in most cases.

SUMMARY

1. First molars of C3H fetal mice were dissected out, pretreated in 30 per cent glycerol, frozen rapidly in liquid nitrogen (–195° C.), rewarmed rapidly at 37° C. in Tyrode's solution, and then transplanted to the anterior eye chambers or axillae of adult mice. Transplants were considered successful when odontoblasts and ameloblasts differentiated and formed dentin and enamel.

2. Tooth germs frozen without pretreatment in glycerol almost always failed to take on transplantation.

3. Pretreatment with glycerol increased the proportion of successful transplants among frozen tooth germs from 0 to 33 per cent in the case of anterior eye chamber transplants and from 4 to 57 per cent in tooth germs transplanted to the axilla.

4. Tooth germs that were exposed to glycerol but not frozen showed less damage than glycerol-treated frozen tooth germs. However, the glycerol solution in itself caused some injury under the conditions of this experiment.

5. The group of tooth germs that were neither treated in glycerol nor frozen showed the highest proportion of successful takes, about 85 per cent in both the anterior eye chamber and axilla.

6. Under the conditions of this experiment, the axilla proved to be more favorable than the anterior eye chamber as a site for transplantation. No difference was noted in the case of control transplants.

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THE COMBINED ANTICARIOGENIC EFFECT OF A SINGLE STANNOUS FLUORIDE SOLUTION AND THE UNSUPERVISED USE OF A STANNOUS FLUORIDE-CONTAINING DENTIFRICE

JOSEPH C. MUHLER

Department of Biochemistry, Indiana University, Indiana University Medical Center, Indianapolis, Ind.

THE anticariogenic effectiveness of the single application technic using stannous fluoride has been demonstrated by Gish, Howell, and Muhler[1] and corroborated independently by Jordan.[2] These observations which were made on children residing in nonfluoride areas have been extended by Muhler in showing the effectiveness of the single application technic in children whose teeth developed in a fluoride area.[3] Similarly, the use of a 10 per cent solution of stannous fluoride applied once each 6 months to young adults has resulted in significantly less dental caries than occurred in a similar group treated with distilled water.[4] The use of a commercially available stannous fluoride dentifrice* has been shown by Jordan and Peterson to be effective after 3 years.[5] The effectiveness of this product has been independently corroborated in a 1-year study by Peffley.[6] Previously conducted in vitro solubility tests, using intact rat molars,[7] have shown that a combination of the single application of stannous fluoride followed by the daily use of a stannous fluoride dentifrice is superior to the use of the single application of stannous fluoride alone. It was of interest, then, to evaluate this combination treatment in a human clinical test.

EXPERIMENTAL

A total of 751 children, ages 6 to 15 years, all of whom resided in Bloomington, Indiana, were included in this study. Each child was given a thorough clinical and radiographic examination, and placed into one of three groups according to his past dental caries susceptibility by methods previously described.[8] The age and sex distribution of the subjects at the initial examination is shown in Table I and the balance of the three groups in terms of average initial DMF surfaces is found in Table II. Group 1 received a single topical application of distilled water and a nonfluoride-containing dentifrice; those in Group 2 a topical application of distilled water plus a stannous

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*Crest brand dentifrice, manufactured by The Procter and Gamble Company, Cincinnati, Ohio.

TABLE I

AGE AND SEX DISTRIBUTION OF SUBJECTS AT INITIAL EXAMINATION

AGE IN YEARS	CONTROL			SnF_2 DENTIFRICE			DENTIFRICE PLUS TOPICAL		
	MALE	FEMALE	TOTAL	MALE	FEMALE	TOTAL	MALE	FEMALE	TOTAL
6	2	5	7	1	5	6	4	7	11
7	6	10	16	12	14	26	5	10	15
8	15	18	33	9	14	23	14	15	29
9	17	13	30	11	19	30	16	20	36
10	15	19	34	27	20	47	20	25	45
11	10	9	19	9	10	19	13	16	29
12	10	11	21	10	14	24	9	14	23
13	11	16	27	7	11	18	9	10	19
14	14	11	25	15	2	17	17	6	23
15	17	4	21	9	7	16	8	3	11
16	3	7	10	3	5	8	5	6	11
17	1	4	5	2	4	6	2	3	5
18	2	0	2	0	2	2	1	1	2
Totals	123	127	250	115	127	242	123	136	259

TABLE II

AVERAGE INITIAL DMF SURFACES OF SUBJECTS STARTING THE TEST

	CONTROL	SnF_2 DENTIFRICE	DENTIFRICE PLUS TOPICAL
Number of subjects	250	242	259
Total DMFS	3,246	3,166	3,225
Average DMFS	12.98	13.08	12.45

fluoride dentifrice. The children in Group 3 received a single topical application of an 8 per cent solution of stannous fluoride and, in addition, received a commercially available stannous fluoride dentifrice. None of the children received any instruction on the use, or the frequency of use, of the dentifrice, but were told only to use it in accordance with their usual oral hygiene practices. The composition of the stannous fluoride dentifrice was identical to that described and used by Jordan[5] and Peffley.[6] All of the children received a thorough prophylaxis and the topical applications were applied immediately thereafter by means of technics already described.[9] At 6-month and 12-month intervals the children were re-examined by the same dentist, radiographed, and received another prophylaxis and topical application.

DATA AND DISCUSSION

The dental caries data obtained in this study are found in Table III. This table shows a comparison of the effect of the various treatments on the incidence of new dental caries at the 6- and 12-month examination periods when expressed in terms of either the DMFT or DMFS indices. These data indicate that 6 months following the initial examinations the group treated topically with distilled water and who used a control dentifrice had a mean increase of 0.86 DMF teeth and 1.97 DMF surfaces. The group receiving the topical application of distilled water and who used, in addition, a SnF_2 dentifrice had a mean increase of 0.53 new DMF teeth and 1.53 new DMF surfaces. These differences represent a reduction in dental caries of 38 and 22 per cent, respectively, when expressed in terms of the DMFT or DMFS indices. The group which received both the topical SnF_2 application and the SnF_2 dentifrice had a

TABLE III

A COMPARISON OF THE EFFECTIVENESS OF A SnF_2 DENTIFRICE OR THE COMBINATION OF THE SINGLE TOPICAL APPLICATION OF SnF_2 AND A SnF_2 DENTIFRICE ON THE INCIDENCE OF NEW DENTAL CARIES 6 AND 12 MONTHS FOLLOWING THE INITIAL EXAMINATION

	6-MONTH EXAMINATION			12-MONTH EXAMINATION		
	WATER TOPICAL PLUS CONTROL DENTIFRICE	WATER TOPICAL PLUS SnF_2 DENTIFRICE	SnF_2 TOPICAL PLUS SnF_2 DENTIFRICE	WATER TOPICAL PLUS CONTROL DENTIFRICE	WATER TOPICAL PLUS SnF_2 DENTIFRICE	SnF_2 TOPICAL PLUS SnF_2 DENTIFRICE
Number of subjects	230	228	233	220	221	230
DMFT increment	0.86	0.53	0.34	1.38	0.94	0.58
Per cent reduction	--	38	61	--	32	58
P =	--	0.085	0.0006	--	0.0048	0.0001
DMFS increment	1.97	1.53	0.69	3.15	2.44	1.31
Per cent reduction	--	22	65	--	23	58
P =	--	0.058	0.0006	--	0.025	0.0001
Reversals in diagnosis	0.20	0.30	0.46	0.15	0.35	0.49

mean increase of new DMFT of 0.34, or a reduction of 61 per cent. When the data are expressed in terms of DMFS index there is an increment of 0.69 or a reduction of 65 per cent. These latter reductions are highly significant statistically, as judged by their low probabilities.

The data obtained 12 months following the initial examinations similarly indicate a significant anticariogenic effect of both the groups using the SnF_2 dentifrice and those receiving the topical SnF_2 treatment plus the SnF_2 dentifrice. When the dental caries experience of the group receiving the topical application of water and the SnF_2 dentifrice is expressed in terms of the DMFT index there is a caries increment of 0.94, or a reduction of 32 per cent. When the DMFS index was used for comparative purposes the increment was 2.44, or a caries reduction of 23 per cent. Both of these differences are statistically significant. The greatest anticariogenic effect resulted in the group which received the combination of topical SnF_2 and the SnF_2 dentifrice. In this group, an increment of 0.58 and 1.31 was found when expressed in terms of the DMFT or DMFS indices, respectively. This is a reduction of 58 per cent in both instances, and these differences are highly significant.

It thus appears that the combination technic using both the single topical application of SnF_2 and, in addition, the SnF_2 dentifrice results in a highly important anticariogenic effect. These data corroborate the previously conducted in vitro studies which suggested the possibility of such an effect. Considerable speculation is possible in order to explain such a result. Of considerable interest in this regard is the finding that, in rats, repeated daily topical applications of the stannous fluoride dentifrice maintains the antisolubility effect produced by the topical application of SnF_2. From a practical preventive dentistry aspect, it will probably be necessary to use all of the

preventive agents available in order to control dental caries, and it is suggested that the way to obtain the maximum effectiveness from either topical stannous fluoride or SnF_2 dentifrice is to use them together. This should not be interpreted as the final word of advice in caries prevention but, at present, the combination approach appears to provide the most effective technic.

SUMMARY

A total of 751 children, ages 6 to 16 years, were divided into three groups in order to test the anticariogenic effectiveness of a SnF_2 dentifrice and a combination of topically applied SnF_2 and a SnF_2 dentifrice. The results after 6 and 12 months showed approximately 30 per cent less tooth decay in the group using the SnF_2 dentifrice alone when the DMFT index was used, and approximately 20 per cent less new dental caries when the DMFS index was used. When the combination of topical and SnF_2 dentifrice was used, approximately 60 per cent less new caries was found after 6 and 12 months by using either the DMFS or the DMFT index. It is suggested that, in order to obtain the maximum anticariogenic effect from the topical SnF_2 application or the SnF_2 dentifrice, both be used in conjunction with each other.

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THE AMINO ACID DECARBOXYLASE OF SALIVARY SEDIMENT

N. GOCHMAN,* R. K. MEYER, R. Q. BLACKWELL, AND L. S. FOSDICK

Department of Chemistry, Northwestern University Dental School, Chicago, Ill.

SEVERAL investigators[1-5] have demonstrated that periodontal disease is accompanied by an above normal rate of putrefaction in the mouth. Amino acid decarboxylation is one of the major pathways of the putrefactive process and, as such, may be studied as an indication of the extent to which putrefaction takes place. The present work is an investigation of the presence and activity of the bacterial amino acid decarboxylases in saliva. A quantitative assay procedure is described which was used to correlate the salivary decarboxylase activity of clinic patients with their periodontal condition. The method is based on Gale's studies[6] of the amino acid decarboxylases of washed suspensions of intestinal bacteria.

MATERIALS AND METHODS

Sample Collection and Incubation.—Fifteen to seventeen milliliters of saliva was collected by paraffin stimulation in heat-sterilized flasks. Fifteen milliliters of the saliva sample was transferred to a sterile screw-cap vial, graduated to indicate the 15 ml. mark. Two and one-half milliliters of sterile 12 per cent (w./v.) casein hydrolysate (Trypticase†) was then added to the vial. The casein hydrolysate solution was prepared in advance and distributed, 2.5 ml. each, into small test tubes. These tubes were stoppered with cotton, sterilized, and kept under refrigeration until required.

The screw-cap vial was placed on a rotating device in a constant-temperature water bath, and removed after 4.0 hours of incubation at 37° C. The contents of the vial were then emptied into a 50 ml. graduated centrifuge tube and centrifuged for 5 minutes at 2,500 r.p.m. The tube was decanted and drained for about 30 seconds on a paper towel. About 5 ml. of distilled water was added to the tube and the sediment stirred into suspension with a glass rod. The tube was centrifuged and drained as before. Another 5 ml. of water was added and the washing procedure repeated. Thorough washing was necessary to remove the residual amino acids from the incubation procedure. The washed sediment was then diluted to the 12 ml. mark with 0.2 M phosphate buffer, pH 6.5, and stirred into a uniform suspension.

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*Present address: G. D. Searle and Company, Chicago, Illinois.

†Baltimore Biological Labs., Inc., Baltimore, Md.

Enzyme Activity Measurements.—All activity measurements were conducted with the use of the Warburg constant-volume respirometer at 37° C. under a nitrogen atmosphere.

One-half milliliter of one of the following amino acid solutions was added to the sidearm of each of five Warburg vessels: 0.08 M L-arginine, 0.16 M D, L-ornithine, 0.08 M L-glutamic acid, 0.08 M L-histidine, and 0.08 M L-lysine. To the main compartment of each flask, 2.0 ml. of the washed, salivary sediment suspension was introduced. The flasks and manometers were flushed with nitrogen for 10 minutes, while being shaken in the 37° C. water bath. After gassing, the manometers were adjusted for gas evolution, and an initial reading was taken following an equilibration period of 2 to 3 minutes. The amino acid solutions were tipped in, and readings taken every 10 minutes for a total of 40 minutes. The enzyme activities were calculated from the CO_2 evolution between 10 and 40 minutes after tipping and expressed as $Q_{CO_2}^{N_2}$: μl CO_2 per hour per 2.0 ml. suspension when prepared as described.

The precision of the method was evaluated by determining the ornithine decarboxylase activity of eight 15 ml. aliquots from a pooled saliva sample. The activities ranged from 138 to 151 with a mean value of 144 and a standard deviation of 5.4.

RESULTS

Development of Standard Assay Procedure.—Preliminary experiments indicated that a washed suspension of sediment from incubated saliva would decarboxylate a mixed amino acid substrate at a slightly acidic pH. Using pooled saliva from various unclassified individuals, experiments were performed to define the properties of these salivary enzymes.

As shown in Fig. 1, optimal decarboxylating activity was developed when the saliva sample was incubated for 6 hours with casein hydrolysate. A 4-hour incubation period was chosen for future experiments as this gave near maximal activity and was more feasible than longer incubation periods.

Incubation of the saliva under varying conditions showed maximum development to occur in the presence of 3 per cent casein hydrolysate, as seen in Fig. 2. Anaerobic conditions or the addition of glucose (2 per cent) during the incubation prevented maximum activity development, while almost no activity appeared in the presence of native casein (3 per cent).

The salivary sediment suspension was next tested for activity on seventeen common amino acids at a reaction pH of 6.0. Only five amino acids gave detectable CO_2 evolution, and their relative activities may be seen in Fig. 3. The L-isomers of alanine, tyrosine, glycine, leucine, isoleucine, aspartic acid, phenylalanine, proline, serine, threonine, tryptophan, and valine were not acted upon under these conditions.

To determine the pH of the optimum activity, the reactions were performed in 0.2 M acetate buffer, pH 5.0 and 5.5, and 0.2 M phosphate buffer,

pH 6.0, 6.5, and 7.0. The activities were corrected for increasing CO_2 retention by the medium, according to the method of Johnson.[7] As shown in Fig. 4, a pH of 6.5 was near the optimum for all five amino acids, and this pH was used in later experiments.

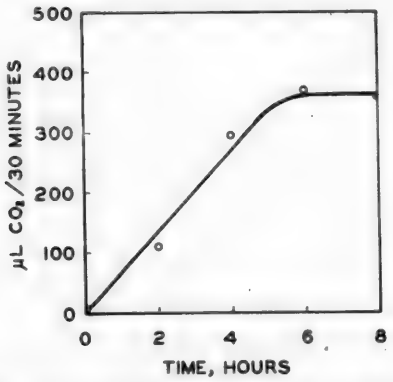

Fig. 1.—The effect of increasing incubation time on decarboxylating activity of salivary sediment suspension. Flask contents: 2.0 ml. 0.2 M phosphate buffer, pH 6.0, containing enzyme preparation, and 0.5 ml. 10 per cent casein hydrolysate in sidearm.

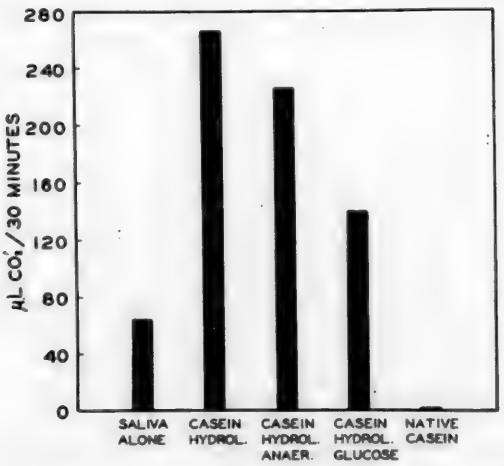

Fig. 2.—The effect of varying incubation conditions on the development of salivary decarboxylase activity. Flask contents: as for Fig. 1.

Assay of Saliva From Clinic Patients.—The individuals used in this study were patients at the Northwestern University Dental Clinic and the Clinical diagnoses were performed by the dental school staff. The patients were classified on the basis of presence or absence, depth, and distribution of periodontal pockets as revealed by visual examination. All other variables, caries, etc., were not considered in this study. Normal patients were those without a manifested

periodontal disease regardless of other conditions. Periodontal patients included all those exhibiting a minor to major periodontal involvement. All saliva samples were collected between 9:30 and 10:30 A.M., and the analyses performed directly after collection.

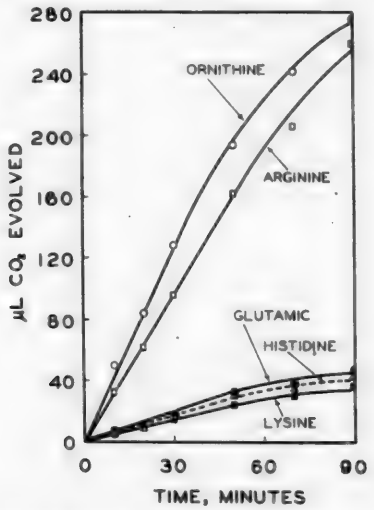

Fig. 3.—Relative decarboxylating activity of salivary sediment toward five amino acids. Flask contents: 2.0 ml. 0.2 M phosphate buffer, pH 6.0, containing enzyme preparation, and 0.5 ml. 0.033 M of the L-amino acids indicated.

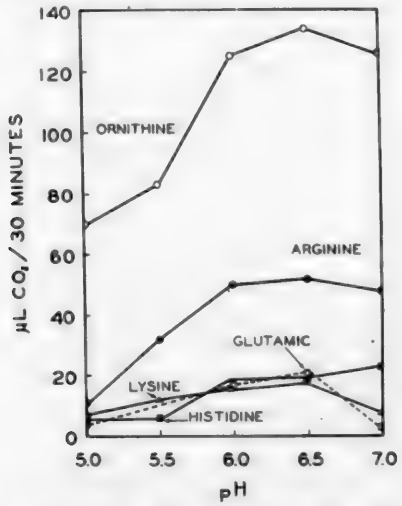

Fig. 4.—The effect of reaction pH on amino acid decarboxylation by salivary sediment.

The results of the assays of saliva from 50 normal subjects and 50 periodontal patients are summarized in Table I, along with the calculated t ratios and P values. It can be seen that a statistically significant difference is present

TABLE I

COMPARISON OF AMINO ACID DECARBOXYLASE ACTIVITY OF NORMAL AND PERIODONTAL SALIVARY SEDIMENT

	ARGININE	ORNITHINE	GLUTAMIC ACID	HISTIDINE	LYSINE	TOTAL
Normal						
$Q_{CO_2}^{N_2}$ ± s.d.	105 ± 48	201 ± 101	32 ± 30	28 ± 23	20 ± 18	387 ± 169
Range	24-216	12-436	0-152	0-108	0-74	70-814
Per cent total	27	52	8.3	7.2	5.2	
Periodontal						
$Q_{CO_2}^{N_2}$ ± s.d.	157 ± 66	274 ± 108	45 ± 45	40 ± 32	32 ± 29	547 ± 223
Range	34-346	78-548	0-232	0-126	0-158	146-1,172
Per cent total	29	50	8.2	7.3	5.8	
t	4.50	3.48	1.70	1.82	2.50	4.03
P	< 0.001	< 0.001	0.1	0.05	0.01	< 0.001

when the decarboxylase activities of the normal and periodontal saliva are compared. The mean periodontal activities were higher in each case than the corresponding normal ones, but this was particularly evident with arginine, ornithine, and the total activity. It should also be noted that if the mean activity for each amino acid is expressed as a per cent of the total activity, the decarboxylases have a well-defined distribution independent of the actual quantitative rates. Thus, arginine decarboxylase accounted for approximately 28 per cent of the total CO_2 evolved in both groups, although the periodontal saliva mean activity was 50 per cent greater. Ornithine decarboxylase was by far the most active of the enzymes in the salivary sediment preparation, accounting for 51 per cent of the total CO_2 evolution.

DISCUSSION

This investigation has produced evidence for the existence of specific amino acid decarboxylases in incubated saliva, which appear to be constituents of the bacterial flora of the mouth. The pH optima of these enzymes was about pH 6.5 and, therefore, they could be active under the physiologic conditions prevailing in the mouth.

It was noted that the individual amino acids were decarboxylated in a specific ratio to one another. This ratio held regardless of how high or low the actual numerical activities were, and was independent of the periodontal conditions. This observation suggests that there is one predominant bacterial species whose particular enzyme distribution and activity were being determined by these measurements. Several organisms have been identified by Gale[6] which have up to five amino acid decarboxylases in their enzyme constitution.

Although the range of activities within each group was very wide, and considerable overlapping existed between groups, the mean activities show a significant increase associated with periodontal disease. The results take on added significance in view of the fact that other variables which might influence the decarboxylase activity were not considered, and the cases used included some in which the periodontal involvement was minor.

SUMMARY

1. The presence of five amino acid decarboxylases was detected in washed suspensions of the sediment from incubated saliva: arginine, ornithine, glutamic acid, histidine, and lysine were acted on.

2. The optimal pH for decarboxylation of these amino acids was about 6.5.

3. A reproducible analytical procedure is described whereby saliva samples may be assayed for their amino acid decarboxylating activity.

4. In a study of the saliva from 50 normal subjects and 50 periodontal patients, the periodontal group showed a statistically significant higher decarboxylating activity for each of the five amino acids tested.

5. It was found that relative activities toward each of the amino acids were similar in all samples, from normal and periodontal saliva. Arginine, ornithine, glutamic acid, histidine, and lysine accounted for about 28, 51, 8, 7, and 5 per cent, respectively, of the CO_2 evolved.

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CULTURE BROTH CONTAMINATION BY RESIDUAL PARA-AMINOTOLUENE SULFONAMIDE (BENZYLOG)

B. F. GURNEY* AND E. JAMES BEST**

Loyola University School of Dentistry, Chicago, Ill.

IN ENDODONTAL therapy, successful sterilization of the root canal is determined with the aid of culture broth. When chemotherapeutic agents are used to attain a sterile field, it is obvious that, in the process of testing for sterility, only the minimum amount of medicament can be allowed to reach the culture broth; otherwise, a false negative result will be obtained.[1]

In a previous work, it was noticed that endodontal bacteria occasionally would grow in broth having concentrations of para-aminotoluene sulfonamide (PATS), as high as 10 mg. per cent.[2] Should this observation hold true for the execution of a routine endodontal culture test, the problem of possible contamination of culture broth with residual PATS would be greatly minimized and an additional advantage for its use in endodontia established. Other advantages of this drug, when used in endodontal therapy, have been presented elsewhere.[3]

It is the purpose of this report to show the relation between carry-over of residual medicament and culture growth inhibition when PATS is used as the endodontal antiseptic.

EXPERIMENTAL METHODS AND RESULTS

Brain-heart infusion broth was made up at concentrations of 0.0 per cent PATS, 1 mg. per cent, and 10 mg. per cent, and at pH 6. Similar solutions were made up at pH 6.5, 7, 7.4, and 8. All culture broths were sterilized by passage through sterile Seitz filters, and incubated at 38° C. for 3 days as a check on sterility.

Positive cultures were obtained from the Department of Endodontia where they had been in incubation for 3 days. Transfers were made to fresh brain-heart infusion broth, and, after 24 hours' incubation, these new growths were used as inoculum. The Gram stain and general morphology of the cultures used in this study are shown in Table I.

A single sterile 3 mm. wire loop was used to transfer bacteria to a sterile microscope slide where the bacterial sample was spread into a thin film. Aseptically, and without delay, a sterile plain endodontal paper point was touched to

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*Dept. of Biochemistry and Research, Loyola University School of Dentistry, Chicago.

**Chairman, Dept. of Endodontia, Loyola University School of Dentistry, Chicago.

TABLE I
CHARACTER OF THE BACTERIAL CULTURES USED IN THIS STUDY

NUMBER OF CULTURES	GRAM STAIN AND GENERAL MORPHOLOGY
5	Gram-positive micrococci
1	Gram-negative micrococci
6	Gram-negative and gram-positive micrococci
1	Gram-positive micrococci; Gram-negative rods
4	Gram-negative streptococci

the bacterial film in such a manner that not more than 1 mm. of it became visibly moist. If the moistened tip of the paper point exceeded 1 mm. the paper point was then destroyed. Frequently the tip was moistened to an extent barely visible. Such bacterial samples were then dropped into tubes of the various culture broths described previously. All determinations were made in duplicate.

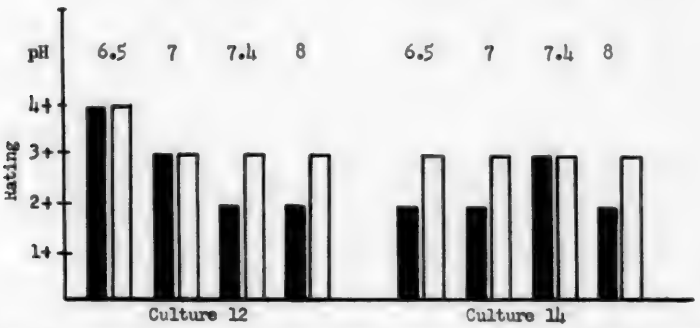

Fig. 1.—Bacterial growth in brain-heart infusion broth with PATS concentration at 10 mg. per cent. *Black bar* = bacterial growth in PATS broth. *White bar* = bacterial growth in control broth.

All culture solutions were then incubated at 38° C. for 72 hours. Observations were made at 24 and 72 hours, although only the 72-hour observations will be reported here. Records were maintained on the basis of 1+ to 4+ judgment of the bacterial growth present. Results of bacterial growth in culture broth with 10 mg. per cent PATS are shown in the bar graph of Fig. 1.

As would be expected, each culture exhibited a different growth pattern; however, all cultures exhibited patterns similar to those shown in Fig. 1. Here, the solid bars represent bacterial growth in culture broth containing 10 mg. per cent PATS at the various pH values of the respective solutions. For comparison, the hollow bars represent bacterial growth in the respective control solutions. Determinations were made at various pH levels, since a previous study had shown that environmental pH played an important role in determining the antimicrobial activity of PATS.[2] This effect, however, was not as apparent at these low concentrations of PATS, since none of the 17 bacterial cultures used here exhibited discernible and reliable variations of growth dependent on the pH of the environment.

A second line of endeavor involving exaggerated contamination of culture broth with PATS was conducted as follows. On each of 10 sterile microscope

slides was placed a sterile PATS impregnated paper point. Each point was then wet thoroughly with sterile 5 per cent PATS solution by means of a cotton pledget previously soaked in the solution, in much the same way as is done in actual practice. After 10 minutes of standing, the impregnated paper points were aseptically removed and discarded. Without delay, a plain paper point was touched to the film of solution on the slide until it was then wet upward from the tip not less than 1 mm. This point was then placed in a sterile vial of plain brain-heart infusion broth. A duplicate broth culture sample was made from this slide. This procedure was repeated for the remaining 9 slides of this experimental series (10 pairs of culture tubes).

Five pairs of culture tubes were inoculated with bacteria from culture A, and the remaining five pairs of culture tubes inoculated with bacteria from culture B (Table II). Each culture tube now contained an exaggerated contamination with PATS and a near minimum of bacterial inoculum. All tubes were incubated at 38° C. for 24 hours, at which time all tubes exhibited bacterial growth. These results, as well as the plan of the experiment, are shown in Table II.

TABLE II

RESULTS FROM SIMULATED, EXAGGERATED CONTAMINATION OF CULTURE BROTH WITH PATS (SEE TEXT FOR EXPLANATION)

	BROTH CULTURE NO. AND PATS IMPREGNATED POINT NO.									
	1	2	3	4	5	6	7	8	9	10
Bacterial inoculum	A	A	A	A	A	B	B	B	B	B
Bacteria	Gram + micrococci					Gram + rods and micrococci				
24 hr. observation	3+	3+	3+	3+	3+	4+	3+	3+	3+	3+

In a third line of endeavor, 257 negative cultures were obtained over a period of time from the Department of Endodontia, all of which were associated with patients undergoing endodontal treatment with PATS as the antiseptic of choice. These negative cultures were inoculated with bacteria in the manner described previously. The results of these inoculations, following incubation, are shown in Table III. It is interesting to note that 5 of the cultures showed no growth of bacteria. This phenomenon has been encountered before and is believed to be due either to a residual bactericidal agent in the paper points or to a binding effect (on the few bacteria present) of the filler in the paper points. It is also possible that the electrostatic charge on the paper points may have rendered the bacteria nonviable. Re-inoculation of the 5 negative cultures by the method described, but using a platinum needle as a transfer agent instead of an endodontal paper point, yielded positive results in all 5 cultures after 72 hours' incubation.

TABLE III.

RESULTS OF INOCULATION OF 257 CLINICALLY NEGATIVE CULTURES

	CULTURE RESULT		
	POSITIVE AT 24 HOURS	POSITIVE AT 72 HOURS	NEGATIVE AT 72 HOURS
Number of cultures	226	26	5

SUMMARY

The problem of residual contamination of culture broth by PATS, as might possibly occur in root canal therapy, was investigated.

Endodontal bacterial growth was not inhibited in brain-heart infusion broth where the PATS concentration was as high as 10 mg. per cent, nor was it inhibited in culture broth with a similated exaggerated contamination of PATS.

Of 257 negative clinical cultures, 98 per cent showed no inhibition to endodontal bacterial growth, provided viable organisms were introduced into them.

It seems clear that of all culture samples taken in the usual fashion during endodontic treatment with PATS, 98 to 99 per cent of the results will be accurate and reliable.

The able assistance of Mrs. Lewellyn Matsumoto is gratefully acknowledged in the execution of this problem.

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THE EFFECT OF HYPERVITAMINOSIS A UPON THE INCISOR AND MOLAR TEETH, THE ALVEOLAR BONE, AND TEMPOROMANDIBULAR JOINT OF WEANLING RATS

ROBERT J. GORLIN AND ANAND P. CHAUDHRY

Division of Oral Pathology, School of Dentistry, University of Minnesota, Minneapolis, Minn.

BECAUSE of the divergence of opinions available on the effect of hypervitaminosis A upon the teeth, periodontium, and temporomandibular joint, the authors thought it worth while to investigate this problem. Irving[1] in 1949 indicated that this vitamin had a minimal effect upon the teeth of rats. No change was observed in the incisor enamel, but the rate of dentin apposition due to odontoblastic atrophy and the amount of interfibrillar cementing substances were reduced. This effect on the mesodermal tissues was also reflected in the lingual alveolar bone where the rate of formation was pronouncedly slowed and a marked decrease in the number of osteoblasts was observed.

Rodahl[4] observed marked hyperemia or hemorrhage in the incisor pulp, as well as in the vacuoles and calcium deposits. An irregular arrangement and occasional degeneration of the odontoblastic layer were noted. The pulpal (inner) dentin manifested amorphous calcification.

On the other hand, Pohto[7] found no changes in the teeth and Wolbach[2] indicated that histopathologic changes in the teeth were inapparent, insofar as remodeling did not occur.

Wolbach[2] in 1947, Maddock and colleagues[3] in 1949, and Rodahl[4] in 1950 performed extensive studies on the general pathologic changes wrought by hypervitaminosis A in the rat, mouse, dog, and other laboratory animals. The most outstanding effects were observed in bone, resulting from the accelerated maturation rate of the skeleton.

Fractures of the long bones were thought to be predicated upon premature closure of the epiphyses and on accelerated substitution of compact for cancellous bone. Anorexia, hemorrhagic diatheses and hypoprothrombinemia, alopecia and exophthalmos were commonly observed. An excellent review of earlier work is that of Rodahl.[4]

Several investigators observed that the bones of younger animals were more rapidly affected[4-6] and Rodahl[4] noted a difference in "acute" and "chronic intoxication" due to vitamin A, the latter producing the far more serious signs.

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He indicated that the absence of normal weight gain was one of the first signs following ingestion of large doses of the vitamin. He attributed this to loss of appetite. In young rats, having a gross consumption of from 500,000 to 1,000,000 I.U., fractures of long bones were manifest from the twentieth to the fortieth day, subsequent to stiffness and limping seen early in the experiment.

MATERIALS AND METHODS

Sixty (60) Sprague-Dawley male rats of weanling age were divided into two groups of 40 experimental and 20 control animals. They were isolated for a week and individually caged. Their initial weights were then recorded. Vitamin A concentrate in corn oil (150,000 I.U. per gram)* was administered daily at a level of 600 I.U. per gram of body weight to the experimental animals and a corresponding amount of corn oil was given to the controls by mouth per syringe. Body weights and clinical data, such as hemorrhage, paralysis, limping, alopecia, and/or diarrhea, were recorded every 3 days. All animals were kept on an ad libitum diet of Purina laboratory chow throughout.

When animals appeared to be terminally ill they were killed by chloroform anesthesia and autopsied. A long bone, costochondral junction, and complete head were placed in 10 per cent formalin for pathologic study. Care was taken to inspect for gross hemarthrosis and subperiosteal hemorrhage at the time of leg removal. The jaws were propped open with applicator sticks for greater ease of penetration of formalin solution.

The bones and teeth were decalcified by the formic acid-sodium citrate method, the skull split longitudinally and returned for further decalcification, and the tissues finally imbedded in paraffin. Specimens were orientated in preparation for sectioning through the long axis of the molar teeth. The incisor tooth preparation was made from the other half of the head. Both longitudinal and cross sections were prepared. All were stained with hematoxylin and eosin by the rapid method.[8]

CLINICAL OBSERVATIONS

By the ninth day, most of the experimental animals were weak and scrubby looking. Perioral alopecia and edema about the eyes were common. Some had frank exophthalmos. Loss of appetite was quite apparent. An abnormal posture with marked bending of the spine was seen. Limping was observed about the twelfth to fourteenth day (in some, by the ninth day) and fractures were apparent in one or more limb bones, usually the hind legs, by the sixteenth to nineteenth day, with hemorrhage about the fracture evident externally. This produced a clinical "paralysis" of the hind limbs.

PATHOLOGIC AND HISTOPATHOLOGIC FINDINGS

The experimental group was considerably smaller than the control animals as may be seen from Fig. 1. Their coat was usually matted and unkempt.

*Supplied by Hoffmann-La Roche, Inc., Nutley, N. J., as synthetic vitamin A palmitate at the concentration of 150,000 units per gram in corn oil. Dilution was made with additional corn oil under nitrogen.

Upon opening the chest and abdominal cavity, injection of lungs and intestinal viscera became apparent. Hemorrhage both intramuscularly and subperiosteally was noted in nearly all animals (Fig. 2).

Fig. 1.—Picture of typical rat on hypervitaminosis A regimen for 12 days. Note size, appearance of coat and eyes. Control littermate is on the right.

Fig. 2.—Subperiosteal hemorrhage adjacent to temporomandibular joint. Hemorrhage was noted beneath periosteum of most long bones. (Orig. mag. ×105.)

In the epiphyseal cartilage of the long bones, the number of cells was reduced, the width of the plate becoming markedly narrower (Figs. 3 and 4). The rate of enchondral bone formation beneath the plate was enhanced, as

manifested by increased depth of calcified cartilage trabeculae. Increased remodeling, demonstrated by large amounts of new endosteal bone and resorption of peripheral bone, was evident. Increased osteoblastic activity was seen,

Fig. 3.—Epiphyseal plate of tibia-control animal. Compare this with Fig. 4 taken at same magnification.

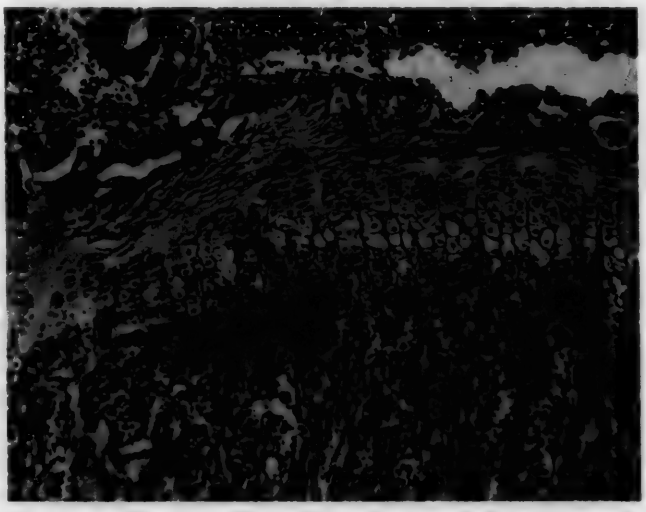

Fig. 4.—Epiphyseal plate of tibia—hypervitaminosis A rat—12 days. Note the reduction in both size and number of cartilage cells. There is evidence of initial closure of the epiphyses. (Orig. mag. ×105.)

especially about the cortical bone of periosteal origin. Animals that were on the vitamin A regimen for a longer time appeared to have an epiphyseal change that indicated closure, as both sides tended to become sealed off with calcified cartilage matrix.

Changes in the incisor teeth were not marked. There was a significant reduction in the thickness of dentin but alteration in the quality of the dentin was not apparent. The odontoblastic layer was reduced in thickness. Increased vascularity and edema of both the pulp and the periodontal ligament were noted, as well as a reduction in the amount of collagen. The thickness of the alveolar bone was significantly reduced (Figs. 5 and 7).

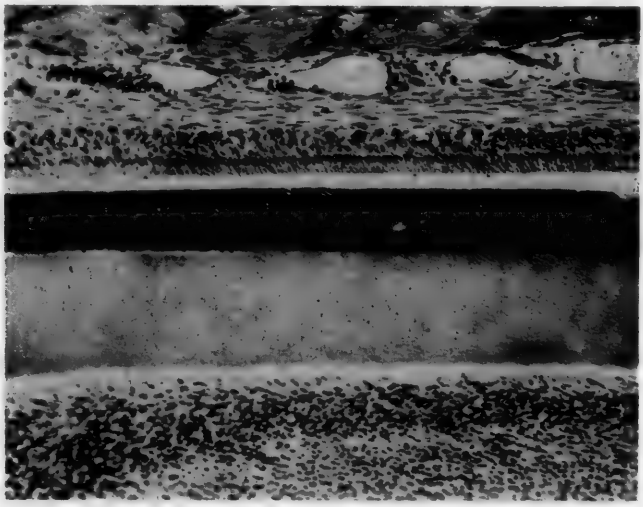

Fig. 5.—Longitudinal section through maxillary incisor—control animal. Compare this with Fig. 6 at same magnification and at same distance from labial alveolar crest.

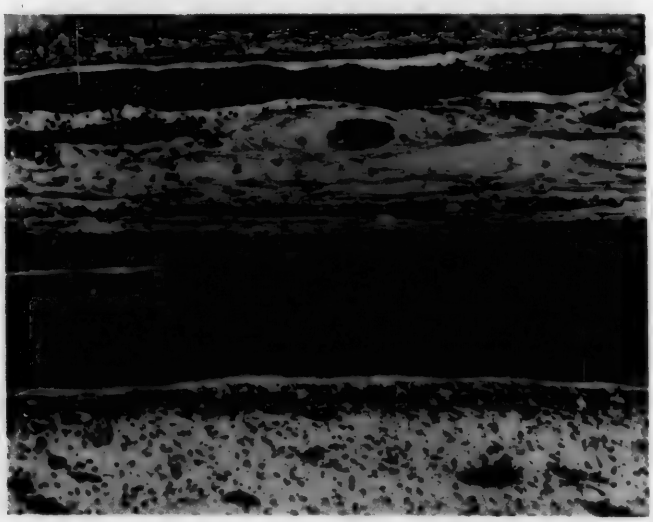

Fig. 6.—Longitudinal section through maxillary incisor—hypervitaminosis A animal. Note decrease in width of dentin, some decrease in the number of odontoblasts. There is a widening of the periodontal ligament. Increased vascularity is apparent both in the pulp and periodontal tissues as well as edema and a paucity of collagen fibers. The entire thickness of the palatal bone is apparent.

Fig. 7.—Pulp of molar tooth of hypervitaminosis A rat. Note marked edema and vascularity of pulp. Odontoblastic layer is intact, however. (×150.)

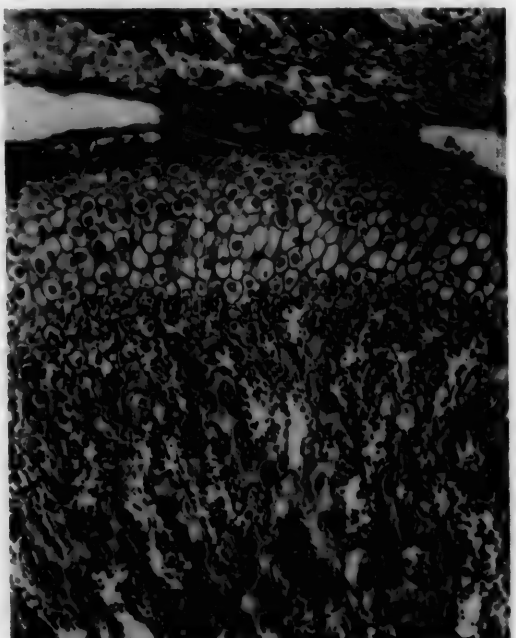

Fig. 8.—Temporomandibular joint—condyle head. Compare this view in the control rat with Fig. 9, taken at the same magnification, ×150.

Fig. 9.—Temporomandibular joint—condyle head—hypervitaminosis A rat. Note marked reduction in size, absence of cartilage cap, numerous fractures of bony trabeculae. Both this and Fig. 7 were made at the same magnification.

Fig. 10.—Callus adjacent to pathologic fracture of ramus of mandible of hypervitaminosis A rat.

The molar teeth showed similar vascular and degenerative changes in the pulp (Fig. 7). There was, however, no apparent alteration in the odontoblastic layer. Cementum deposition was also reduced.

The most marked changes were apparent in the temporomandibular joint. The condylar head was greatly reduced in size and its architecture altered. Subperiosteal hemorrhage was present in 78 per cent of the experimental group.

The cartilage cap was not present and numerous fractures of bony trabeculae were noted (Figs. 8 and 9). In one animal, complete pathologic fracture of the ramus was noted with resultant callus formation. No difference was noted in the thickness of the gingival epithelium of the two groups.

DISCUSSION

Hypervitaminosis A has far less effect upon the teeth than upon the bone. Loss of appetite is part of the toxic reaction induced by the large amount of vitamin A. As is true in many induced single vitamin "deficiencies," loss of appetite results in deprivation of not only other vitamins and minerals but protein as well. Changes in the joints, the teeth, and the periodontium in starvation and in protein deficiency have been studied by several investigators and serve as a base line.[9-12] Most of the changes seen in the bones and teeth in hypervitaminosis A may be predicted upon protein deficiency. Increased vascularity and edema of both the pulp and periodontal ligament seem to be the only *specific changes* seen in this condition.

SUMMARY

Hypervitaminosis A had severe effect upon the temporomandibular joint but minimal effect upon the teeth. Reduction in the amount of dentin, the odontoblastic changes, and the decrease in collagen of the periodontal ligament can be due to loss of appetite. Marked vascularity of both the pulp and periodontal ligament appeared to be the only specific changes in this condition.

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AN ORAL FILAMENTOUS MICROORGANISM: CULTURAL CHARACTERISTICS AND MICROBIAL RELATIONSHIPS AFFECTING GROWTH

ROBERT L. RICHARDSON AND JEAN SCHMIDT

Department of Bacteriology, College of Medicine, State University of Iowa, Iowa City, Iowa

IN A PREVIOUS report from this laboratory, a salivary census was presented for an organism tentatively identified as a leptotrichia.[1] Subsequently, the organism was found to be similar to *Leptotrichia buccalis*, described by Thjøtta, Hartmann, and Bøe,[2] and Hamilton and Zahler.[3] However, the organism described by these investigators is not the only one proposed as leptotrichia or leptothrix. An entirely different organism was characterized and named *Leptothrix buccalis* by Bulleid.[4] Since that study, Bibby and Berry (Type I),[5] Bartels,[6] Gilmour and Hunter,[7, 8] Howell and Rogosa,[9] and others[10, 11] have isolated and described organisms similar to the one characterized by Bulleid.

Through the courtesy of Dr. Marion Gilmour, of the Eastman Dental Dispensary, cultures of the Bulleid-type filamentous organism were obtained for study, and, concurrently, an attempt was made to isolate the organism from specimens of saliva. When the organism had not been isolated after several trials, saliva was heavily seeded with a pure culture suspension of an organism obtained from Dr. Gilmour, and the mixture was plated. Typical colonies of the filamentous organism could not be found on these plates, and stained preparations of the growth failed to exhibit filamentous forms. Control plates inoculated with only a saline suspension of the filamentous organism showed good growth. Thus, it appeared that either the filamentous organism had grown and undergone a marked morphologic change, or that growth had been inhibited by saliva.

The purpose of this paper is to characterize the strains of the filamentous organism subsequently isolated from plaque material, and to report that growth of the organism was inhibited by oral streptococci and stimulated by oral staphylococci.

METHODS

The method used to isolate the filamentous organism was essentially that recommended by Gilmour and Hunter[8] and Gilmour.[12] The isolation medium contained 5.2 per cent brain-heart infusion agar (Difco), 0.5 per cent glucose,

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and 0.2 per cent yeast extract (supplemented infusion medium). Plaque scrapings from one or two tooth surfaces were emulsified in 0.5 ml. saline and diluted 1 to 10 and 1 to 100. Two spread plates for each concentration of the specimen were prepared; the first plate received an inoculum of 0.1 ml. which was spread over the medium using a bent glass rod, the second plate was inoculated with only that inoculum remaining on the rod following preparation of the first plate. To prevent dehydration of the medium, inoculated plates were wrapped in plastic bags or placed in closed jars prior to incubation in air at 37° C. After 4 to 6 days' incubation the surface growth was examined using a dissecting microscope. Colonies thought to be those of the filamentous organism were spaded from the medium and streaked upon 5 per cent sheep blood agar. Pure cultures were obtained by repeatedly streaking isolated colonies. Stock cultures were transferred weekly on slants of supplemented infusion medium without glucose, and on blood agar.

Several physiologic characteristics were determined for the 10 strains (Nos. 1-10) isolated in this laboratory and 2 strains (No. 18, No. 47) obtained from Dr. Gilmour. The tests were performed or read after 7 days' aerobic incubation at 37° C., except for the fermentations which were observed through 14 days. The capability of the strains to ferment 20 carbohydrates was examined. The basal broth used in the fermentation tests contained 1.0 per cent tryptose, 0.2 per cent yeast extract, and phenol red indicator, and was adjusted to pH 7.4. Carbohydrates, prepared as 10 per cent solutions, were sterilized by autoclaving for 10 minutes and were added to tubes of the basal broth to provide a 1.0 per cent concentration. The reduction of nitrate and the hydrolysis of starch, gelatin, and urea were determined on brain-heart infusion agar containing, respectively, 0.1 per cent KNO_3, 1.0 per cent starch, 3.0 per cent gelatin, and 1.5 per cent urea. Phenol red indicator was added to the medium used in the test for urease activity; the rate of color change in medium containing urea was compared to that which the organism produced in the same medium without urea. Methyl red and Voges-Proskauer reactions were determined in MR-VP medium (Difco), and indole and H_2S production in SIM medium (Difco). The pH growth range and extent of growth in the presence of various concentrations of sodium chloride were tested in brain-heart infusion broth supplemented with 0.2 per cent yeast extract.

To assay the inhibition of growth of the filamentous organism, a double layer plate was used in which the test organism was contained in only the top layer. Substances being screened for inhibitory activity (usually a saline dilution of saliva) were applied to the surface of the test plates in one of the following ways: (1) 0.2 ml. of the agent was deposited into stainless steel cylinders (8 mm. diameter) set upon the surface of the medium, (2) sterile filter paper disks (12.5 mm. diameter, No. 740-E, Schleicher and Schuell) were dipped into the agent and placed on the plates, (3) 0.05 ml. of the agent was dropped at the edge of plated medium and permitted to flow across the diameter of the plate. To prepare the double layer plates, 10 to 12 ml. of sterile supplemented infusion medium was poured into sterile Petri plates and allowed to solidify.

Next, growth from one supplemented infusion slant was suspended in 10.0 ml. of saline and emulsified by vigorous pipetting. After the heavier bacterial clumps had settled, 1 to 2 ml. of the upper most portion of the suspension was inoculated into 100 ml. of medium at 50° C.; 6 to 7 ml. of this inoculated medium were then poured over the bottom layer of sterile agar. Since strain No. 18 emulsified more completely than other strains, it was used most frequently as the test organism. All strains, however, served as the test organism at least once.

Fig. 1.—Portion of a primary isolation plate showing (*arrows*) colonies of the filamentous organism. Medium was brain-heart infusion agar supplemented with yeast extract. Plate was incubated aerobically at 37° C. for 4 days. The wrinkled colony with scalloped edge was seen more frequently on isolation plates than the convex colony with fuzzy edge.

RESULTS

Isolation and Characterization of the Filamentous Organism.—Attempts to isolate the filamentous organism from 20 specimens of saliva were unsuccessful. However, 10 strains were isolated from 27 specimens of plaque material. Isolations were made only on plates prepared from the undilute suspension and the 1 to 10 dilution of the specimen. Seldom were more than 10 colonies of the filamentous organism seen on isolation plates; they were invariably hidden among several hundred colonies of other microorganisms. To locate colonies of the filamentous organism, the growth had to be inspected carefully under magnification. In retrospect, it is felt that isolations would have been more plentiful if glucose had been omitted from the isolation medium.

Two colony types were found on isolation plates (Fig. 1); both were 0.5 to 1.0 mm. in diameter, and light tan to white in color. One type had a convex

elevation and a fuzzy edge caused by the outward spread of filamentous cells; it had the appearance of a circular matt with frayed edge (Fig. 2, *a* and *b*). The other colony type exhibited a scalloped edge and wrinkled surface (Fig. 2, *d*); it usually showed a limited filamentous cell formation beneath the surface of the medium.

Pure cultures of the organism also exhibited both the matt and wrinkled colonies on either blood or supplemented infusion agar. However, blood agar supported the matt colony growth to advantage and supplemented infusion agar favored development of the wrinkled colony.

Fig. 2.—Colonies of the filamentous organism following 4 days aerobic growth on brain-heart infusion agar supplemented with yeast extract. Scale shown in "*a*" also applies to *b*, *c*, and *d*. The photographs are of different colonies in different stages of growth.

a, Low convex colony with filamentous edge. This is the matt-type colony.

b, Matt colony with raised edge and a slightly concaved surface.

c, Matt colony starting to exhibit folds and wrinkles.

d, Wrinkled colony with scalloped edge.

The growth on blood agar was quite adherent as much of the colony developed within the substratum of the medium. Colonies on blood agar were white to gray in color, had a solid nonyielding consistency, and were nonhemolytic. Growth from blood or supplemented infusion agar was difficult to emulsify.

All strains isolated were morphologically similar to organisms described by Bulleid,[4] Bibby and Berry (Type I),[5] Bartels,[6] Gilmour and Hunter,[7, 8] and

Howell and Rogosa.[9] Although the main cellular structure consisted of Gram-positive filaments (1 μ by 20 to 100 μ), the unique and distinctive structures were the whip-like and hockey stick-like forms which occurred rather frequently in all strains (Fig. 3, *a*, *b*, *c*). Independent rods, which measured 10 to 15 μ in length, were also found scattered about the filaments and whip forms; fragmentation of filaments presumably furnished these rods. Filaments were nonsegmented, although infrequently, 3 or 4 bacillary bodies were seen in alignment. Dichotomous branching was observed in most strains (Fig. 4). The organisms were

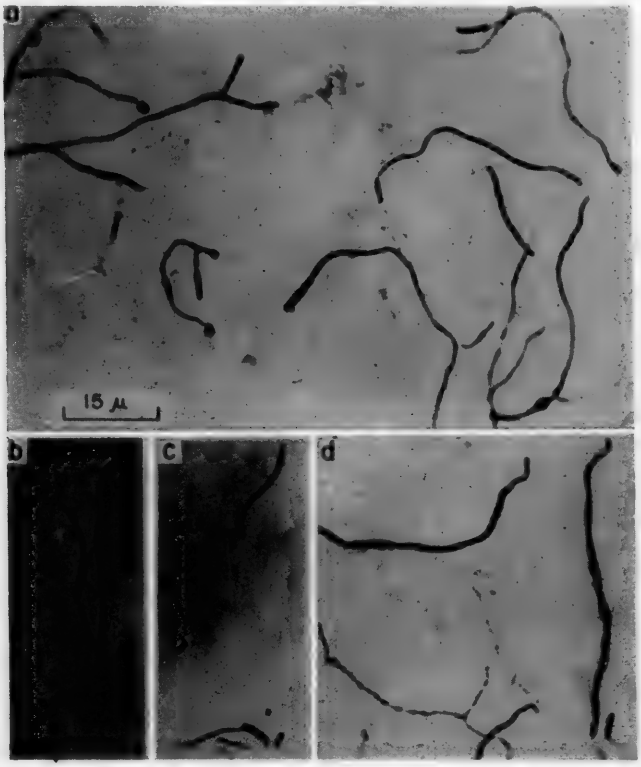

Fig. 3.—Gram stain preparations from 5 day aerobic growth on supplemented infusion agar. Scale in "*a*" also applies to *b*, *c*, and *d*.

a, General morphology of the filamentous organism. Notice the uneven width of the filaments, whip form (*upper right*), branching, septation (*upper left*), and bizzare "y" form.

b, Filaments featuring the distinctive whip-like structure.

c, Hockey stick-like forms or small whip-like structures.

d, Densely staining bodies in filaments affording a beaded appearance.

not acid-fast when stained by the Ziehl-Neelsen method,[13] although a few granules in some filaments appeared to retain carbol fuchsin. Metachromatic granules were demonstrated by Laybourn's stain.[13]

All strains were catalase positive, Voges-Proskauer positive, hydrolyzed starch, and reduced nitrate to nitrite. None of the strains produced H_2S, formed indole, or hydrolyzed gelatin; none exhibited urease activity. Methyl red reactions were negative for 6 strains and doubtfully positive for 6 strains. Although

moderate growth occurred on mycological agar (Difco) at 37° C., none occurred on Sabouraud dextrose medium (Difco) at either 37° C. or 26° C. There was no growth on blood or supplemented infusion agar at 26° C. No change was observed in litmus milk. All strains feremented glucose (no gas), sucrose, maltose, mannose, dextrin, and salicin. Raffinose was slowly fermented by 3 strains. None of the strains fermented arabinose, dulcitol, galactose, inositol, inulin, lactose, mannitol, melezitose, melibiose, rhamnose, sorbitol, trehalose, or xylose. The glucose fermentation broths varied between pH 4.9 and 5.5 after 14 days' incubation. Growth was restricted to medium having a pH of from 5.8 to 8.4. In broth containing 2 per cent sodium chloride, growth was poor.

Inhibition and Stimulation of Growth of the Filamentous Organism.—To determine the effect of saliva on growth of the filamentous organism, tenfold serial dilutions of saliva from 4 subjects were applied to double layer test plates

Fig. 4.—Unstained wet preparation of the filamentous organism showing branching. Preparation was made from 5 day aerobic growth on supplemented infusion agar.

by the cylinder, disk, and drop methods. Plates were examined after 4 days' aerobic incubation at 37° C. It became apparent that dilutions of saliva as high as 1 to 1,000 would usually produce inhibitory zones of from 2 to 5 mm. about the cylinders and disks (Fig. 5) on supplemented infusion medium. Occasionally, a 1 to 10,000 dilution had inhibitory activity. Comparable degrees of inhibition occurred under anaerobic environment. Seitz-filtered saliva had no inhibitory effect on growth of the filamentous organism.

Bacterial antagonism was suggested as the mechanism involved, when zones of inhibition were observed about growth from inocula which leaked from some cylinders and disks during the saliva titration experiments. From the rims of disks exhibiting inhibition, inocula were obtained and streaked on other test plates. Colonies which appeared to inhibit the test organism on these plates were restreaked to obtain pure cultures. By this method, 41 strains of oral bacteria which inhibited growth of the filamentous test strains were isolated from saliva of 4 human beings. Of these 41 strains, 38 were identified as streptococci and 3 as staphylococci. Of the streptococci, 19 strains were *Streptococcus*

salivarius, 8 were *Streptococcus mitis,* and 11 strains could not be readily identified. All 3 strains of staphylococci were *Staphylococcus epidermidis.*

Thompson and Johnson[14] reported that certain oral streptococci produced sufficient hydrogen peroxide to inhibit growth of *Corynebacterium diphtheriae.* The possibility that the filamentous organisms were being inhibited by the same mechanism was investigated. Of 7 inhibitory streptococci tested none produced H_2O_2 in stationary cultures, and only 3 strains produced H_2O_2 in low concentration (10 to 20 μg/ml.) in vigorously aerated cultures. From these experiments it seemed unlikely that H_2O_2 was causing the inhibition of the filamentous organism.

Acid production by the inhibitory organisms was given consideration as the mechanism causing the inhibition since the filamentous organism had been unable to initiate growth in medium below pH 5.8. For these studies the

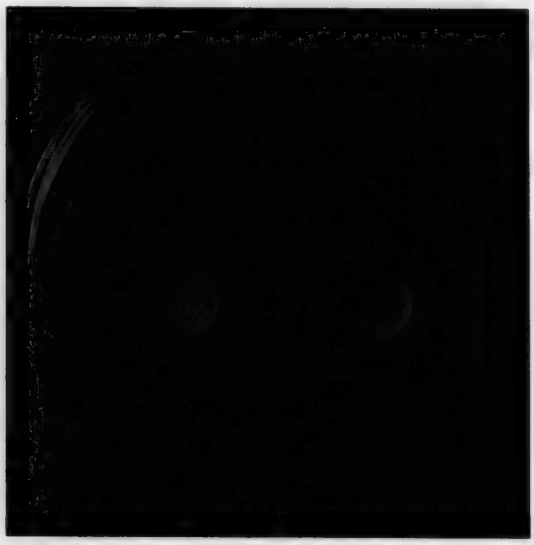

Fig. 5.—Pour plate growth of filamentous organism on supplemented infusion medium showing zone of inhibition about filter paper disk at *left* which had been dipped into a 1/100 dilution of saliva and placed on surface of medium at time of incubation; disk on *right* had been dipped into 0.85 per cent sterile saline.

streptococci used in the H_2O_2 experiments were grown for 3 days in supplemented infusion broth. At the end of this time most strains had lowered the pH of the broth from 7.4 to 4.6. The acidity produced was such that 1.1 to 1.2 ml. of 0.5 N NaOH was required to neutralize 8.0 ml. of broth. Each culture was passed through a Seitz filter and each sterile filtrate divided so as to provide an acid filtrate fraction, and a second fraction which was neutralized with sterile 0.5 N NaOH. Quantities of 0.5, 1.0, and 2.0 ml. of both the acid and neutralized filtrates were dispensed in separate test tubes and brought to a constant volume of 2.0 ml. by the addition of sterile saline; tubes containing sterile broth and saline in similar volumes were included as controls. Three

milliliters of supplemented infusion medium, inoculated with the test filamentous organism, were pipetted into all tubes and the tubes incubated for 7 days. Each day, during the incubation period, all tubes were examined for growth. The results of these experiments indicated that acid production by the streptococci was sufficient to inhibit the test organism, for no growth occurred in any of those tubes which contained 2.0 ml. of acid filtrate. Good growth of the test organism was obtained in all control tubes, tubes containing neutralized filtrates, tubes containing 0.5 ml. of acid filtrate, and most tubes containing 1.0 ml. of acid filtrate.

The 41 strains of streptococci and staphylococci were examined for their ability to inhibit growth of 11 other strains of filamentous microorganisms on supplemented infusion medium. All of the streptococci and staphylococci inhibit growth of all 11 filamentous organisms to some degree.

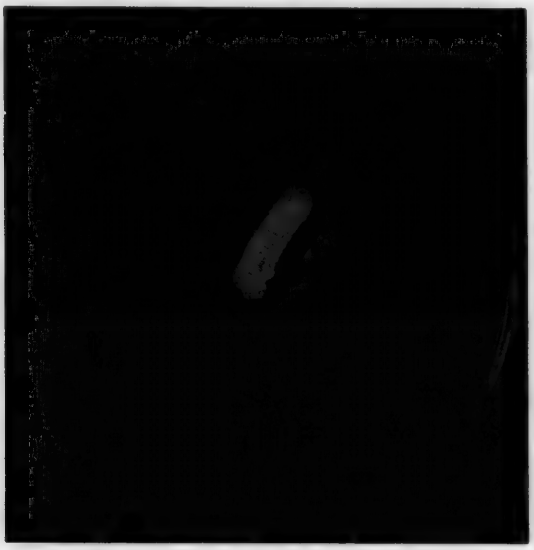

Fig. 6.—Pour plate growth of the filamentous organism on nutrient agar showing zone of growth-stimulation surrounding the streaked *Staphylococcus aureus*.

Since acid production by streptococci caused the inhibition, it seemed reasonable to expect the degree of inhibition to vary directly with the concentration of carbohydrate in the medium. To test this hypothesis, pour plates of the filamentous organism were prepared using supplemented infusion medium without the added glucose, and with 0.5 and 1.0 per cent glucose. Growth of the filamentous organism about saliva-soaked disks placed on the surfaces of these media was compared. As anticipated, the inhibitory zones produced on the medium containing 1.0 per cent glucose were larger by several millimeters than those produced on the medium containing 0.5 per cent glucose. It was noteworthy that on medium not containing added glucose, zones of inhibition were only occasionally produced.

Surrounding some of the disks on the medium not supplemented with glucose was a 3 to 5 mm. band of denser growth of the filamentous organism. This stimulatory effect about the saliva-soaked disks was most frequently demonstrable with a small inoculum which furnished approximately 80 colonies per plate. A large inoculum of the filamentous organism obscured the stimulation.

The stimulatory effect recalled the satellite phenomenon of *Hemophilus influenzae* about "V" producing *Staphylococcus aureus*. Preliminary tests using a stock *Staph. aureus* showed this organism to be capable of stimulating growth of the filamentous organism on supplemented infusion medium without added glucose and on nutrient agar. The stimulatory zones of growth were quite pronounced on nutrient agar.

From the salivas of 9 human beings, 5 strains of *Staph. aureus* and 9 strains of *Staph. epidermidis* were isolated. All these staphylococci promoted satellite growth of several *H. influenzae*. Each staphylococcal strain was examined for its effect on growth of 12 strains of the filamentous organism on nutrient agar. These tests showed 12 of the 14 staphylococci to be effective growth stimulators of all the filamentous organisms (Fig. 6). Two strains of *Staph. epidermidis* failed to stimulate growth and were inhibitory.

DISCUSSION

At present there is no evidence for the streptococcal inhibition and staphylococcal stimulation of the filamentous organism in vivo as described in these in vitro studies. It would be interesting to investigate this subject for, from such a study, one would probably obtain information concerning some of the microbial interrelationships involved in calculus formation.

Bulleid[4] demonstrated that his filamentous organism evoked the precipitation of calcium phosphate when it was grown in nutrient broth which contained added calcium chloride. Also, he achieved some success in provoking deposits of tartar in cats by inoculating the organism into areas where the gingival tissue had been reflected. Following that study, Bulleid stated,[15] "This organism is found wherever there is a deposition of tartar around the teeth, and there seems to be sufficient evidence for the statement that without its presence there would be no tartar formation."

Information concerning this filamentous organism indicates that it is involved in calculus formation, that it fails to grow in medium having a pH below 5.8, that streptococci and probably other acidogenic organisms inhibit its growth by fermenting carbohydrates and establishing too acid an environment, and that many staphylococci elaborate a factor which will stimulate growth of the organism. This information suggests that microbial interactivity may influence the formation of calculus. In this instance the microbial interassociations which are suggested as perhaps important in calculus formation are (1) the size of the population of acidogenic organisms in the precalculus plaques, (2) the frequency and duration with which acid conditions are established within these

plaques, and (3) the presence within these plaques of staphylococci which produce the growth-stimulating factor. These ecological determinants would presumably control the population size and growth of the calcium-precipitating filamentous organisms and in doing so would influence the formation of calculus.

Since the streptococcal inhibition was due to acid production, it would seem reasonable to expect this inhibition to function in vivo. However, had the streptococcal inhibition been demonstrated to be due to hydrogen peroxide, the in vivo operation of the inhibition would be quite questionable. Kraus, Nickerson, Perry, and Walker[16] could not detect even 1 μg of peroxide per milliliter of saliva, and concluded that the inhibitory effect of extracellular biogenic peroxide was either slight or absent in vivo.

It is of interest that Bibby, Hine, and Clough[17] tested the inhibitory activity of saliva on 3 strains of leptotrichia (and many other organisms), and found none of the 3 strains to be inhibited by saliva.

It should be emphasized that the filamentous organism used in our study is not the only organism which has been proposed as *L. buccalis*. The *L. buccalis* described by Thjøtta, Hartmann, and Bøe,[2] and more recently by Hamilton and Zahler,[3] assumed the form of a filament through alignment of 2 to 4 rods, each rod measuring 6 to 10 μ. The organism, as described by these investigators, stains Gram positive when young, is microaerophilic, does not branch or form indole, usually fails to reduce nitrate, lacks catalase, and ferments a number of carbohydrates without production of gas. To a considerable extent the *L. buccalis* described by Thjøtta[2] and Hamilton and Zahler[3] complements the limited description presented for *L. buccalis* in the fifth edition[18] and preceding editions of *Bergey's Manual of Determinative Bacteriology*. Quite similar organisms described by Jay,[19] and thought to be leptotrichia, have been recently assigned the scientific name *Catenabacterium leptotrichoides*.[20]

Since 1948 Bergey's Manual has regarded the genus *Leptotrichia* as an invalid taxonomic group.[21] Such action was taken because early descriptions of the genus were so inadequate that identification of the type species *(L. buccalis)* was uncertain. Nevertheless, the name is valid. The Judicial Commission of the International Committee on Bacteriological Nomenclature and Taxonomy recently offered the opinion[22] that the genus name *Leptotrichia* Trevisan 1879, with the type species *Leptotrichia buccalis* (Robin) Trevisan 1879, be conserved.

In an attempt to resolve the confusion concerning nomenclature, Davis and Baird-Parker[23] proposed that the organism of the type described by Thjøtta be assigned the name *L. buccalis*, and that the organism described by Bulleid be named *L. dentium*. The family to which the genus would be placed was not mentioned. Although Davis and Baird-Parker called attention to the marked morphologic and physiologic differences between these organisms, they considered the inclusion of both species in the same genus reasonable from the standpoint of taxonomy. The Davis and Baird-Parker proposal has not been adopted in this paper because it fails to provide the appropriate taxonomic status for either organism.

We contend that if one follows the classification system used in Bergey's Manual,[20] the Thjøtta-type organism will key into *Eubacteriales, Lactobacilleae,*

and into either the genus *Lactobacillus* or *Catenabacterium;* the Bulleid-type organism keys into *Actinomycetales,* and *Actinomycetaceae.* While the placement of the Bulleid-type organism in either the genus *Nocardia* or *Actinomyces* is not entirely satisfactory, evidence at present seems to indicate that assignment to genus *Nocardia* would be preferred. The reasons for favoring assignment to Nocardia are that the organism is catalase positive, can be isolated on plates incubated aerobically, and hydrolyzes starch. For assignment to the genus *Actinomyces* these three characteristics would have to be different. The Bulleid-type organism cannot be grouped with Nocardia without reservation because it is not an obligated aerobe, its filaments are wider and longer than those of many species of Nocardia, and the characteristic whip-like structures which the organism forms is not common with species of Nocardia. Minor points wherein the organism lacks typical Nocardia characteristics are failure to grow at room temperature, and to form coccal elements by fragmentation. Thus, for the most suitable taxonomic placement of the organism described by Bulleid, it may be necessary to establish a new genus in the family *Actinomycetaceae.*

SUMMARY

Ten strains of a Gram-positive filamentous organism which formed unique and characteristic whip-like structures were isolated from plaque material. All strains were morphologically similar to *Leptothrix buccalis,* described by Bulleid. Biochemically, the organism was catalase positive, Voges-Proskauer positive, hydrolyzed starch, reduced nitrate to nitrite, fermented a number of carbohydrates without gas production, and failed to grow in medium having a pH below 5.8 or above 8.4.

A 1 to 1,000 dilution of unfiltered saliva inhibited growth of the organism on brain-heart infusion agar supplemented with glucose and yeast extract. The inhibition resulted from a bacterial antagonism. *Streptococcus salivarius, Streptococcus mitis,* and *Staphylococcus epidermidis* inhibited growth of the filamentous organism. Acid production by the streptococci and staphylococci caused the inhibition.

When the filamentous organism was grown on nutrient agar, or on brain-heart infusion agar supplemented with only yeast extract, stimulatory zones of growth were frequently produced around saliva-soaked disks placed on the surface of the medium. Several strains of *Staphylococcus aureus* and *Staphylococcus epidermis,* isolated from saliva of human beings, caused a pronounced stimulation of growth of the filamentous organism on nutrient agar.

We want to thank Dr. Marion Gilmour, of the Eastman Dental Dispensary, for her personal communication concerning isolation of the organisms and for the strains which she so graciously forwarded. Also, we want to acknowledge our appreciation to Mr. Frederick W. Kent, who prepared the photographs presented in this paper.

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ADDITIONAL STUDIES ON THE SOLUBILITY OF CERTAIN DENTAL MATERIALS

RICHARD D. NORMAN, MARJORIE L. SWARTZ, AND RALPH W. PHILLIPS

Department of Dental Materials, Indiana University School of Dentistry, Indianapolis, Ind.

IN a previous publication the solubility of silicate and zinc phosphate cements in dilute organic acids, of varying pH, has been reported.[1] This research has been continued to include certain materials not previously studied and to determine the effect of additional variables on the solubility of silicate cement.

The procedure was the same as that employed in the earlier investigation.[1] Discs of the particular material were suspended in distilled water and buffered dilute acids for varying periods of time. Generally only those media were used which had been found previously to be the most deleterious: acetic acid, pH 4.0; citric acid, pH 4.0; and lactic acid, pH 4.0. Distilled water, pH 6.0, was also included since this is the common laboratory test medium employed in cement solubility tests. As in the former study, the organic acids were 0.001 M solutions buffered with their sodium salts. The storage times were 24 hours and 1 week in the same solution and 1 week with the solutions being changed daily, although some tests were also run with silicate over a 28-day period. A minimum of four specimens were used for each set of conditions and the mean values are presented. The solubility of individual specimens varied less than 0.5 per cent. The dental materials studied may be seen in Table I.

TABLE I
MATERIALS USED IN THIS STUDY

MATERIALS	MANUFACTURERS
A. Silicate	
De Trey's synthetic porcelain	The L. D. Caulk Company
B. Zinc-silicate	
Kryptex	The S. S. White Dental Mfg. Co.
C. Resins	
Grip cement	The L. D. Caulk Company
Kadon	The L. D. Caulk Company
PF	American Consolidated Mfg. Co., Inc.
D. Cavity liners	
Copalite	Harry I. Bosworth Company
Handi-liner	Mizzy, Incorporated
Repelac	The L. D. Caulk Company
E. Zinc oxide and eugenol (USP grade)	Mallinckrodt Chemical Works

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RESULTS

Silicate Cement.—Additional studies with silicate cement were performed in order to investigate the effect of manipulative variables as well as the effect of citric acid, pH 4.0, on the continued rate of dissolution. In previous research it was noted that when the solutions were changed daily the rate of dissolution of silicate specimens in acetic acid, lactic acid, and distilled water decreased at the end of the second or third day. However, this gradual de-

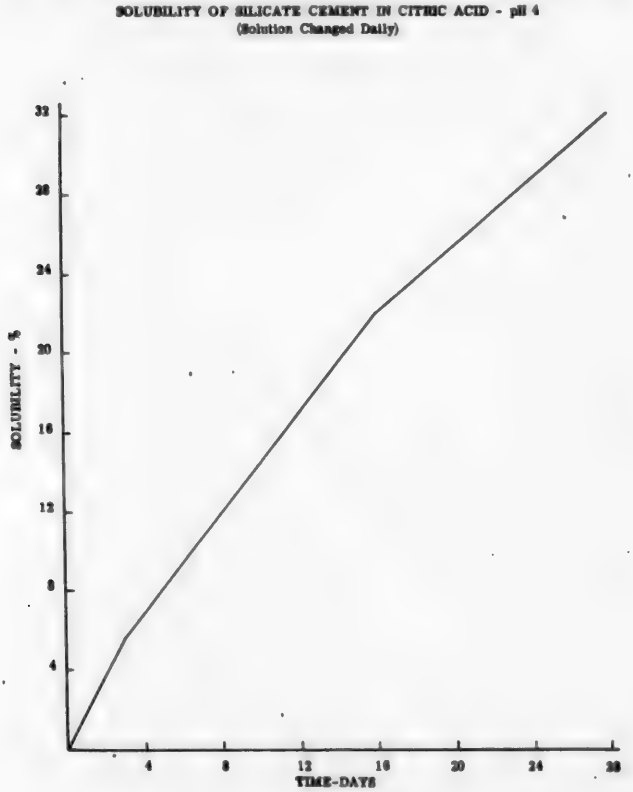

Fig. 1.

crease in solubility with time was not observed in tests made with citric acid. As can be seen in Fig. 1, citric acid, pH 4.0, continued to produce marked dissolution of the silicate cement after 28 days.

Although the surface area of these specimens was not altered greatly, it was necessary to increase the weight of the discs in order that the experiment could be conducted over this extended time interval. Because of this, the percentages which appear in these data (Fig. 1) are lower than those given elsewhere. The actual gram weight losses, however, were comparable to those obtained in the other tests with this material.

The effect of polishing and protecting the silicate restoration was investigated (Figs. 2 and 3). Three series of specimens were employed. The first involved the use of discs which were not protected by cocoa butter or polished. In the second series, the surface was unprotected but polished, while the third series was both protected and polished. All discs were fabricated at the same time and allowed to set for 1 hour in 100 per cent humidity after the start of the mix. The discs were then weighed and divided into three groups of four discs

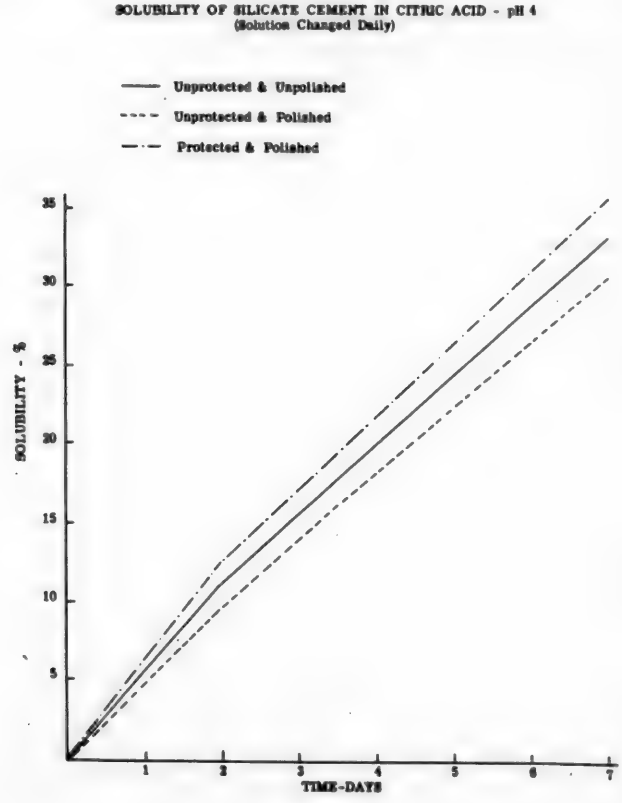

Fig. 2.

each. On removal from the humidor, the first specimens, the unprotected and unpolished, were placed in the test solutions. The second series was unprotected but polished by means of a sandpaper disc and flour of pumice, care being taken not to overheat the silicates. This was done immediately after the 1-hour storage period and the discs were then placed in the test media. The surfaces of the third series were afforded the protection of cocoa butter and were allowed to remain in the humidor for an additional 24 hours. At the end of this time, the cocoa butter was removed by means of an organic solvent, the discs polished and placed in the test solutions. Although variations were

noted in the behavior of individual specimens and solutions, there appeared to be little effect on solubility as influenced by these variables.

Altering the powder-liquid ratio caused no marked variation in solubility when the specimens were stored in citric acid, pH 4.0 (Table II). On the other hand, the effect of a low powder-liquid ratio was to increase dissolution when specimens were stored in distilled water, pH 6.0. Although the actual

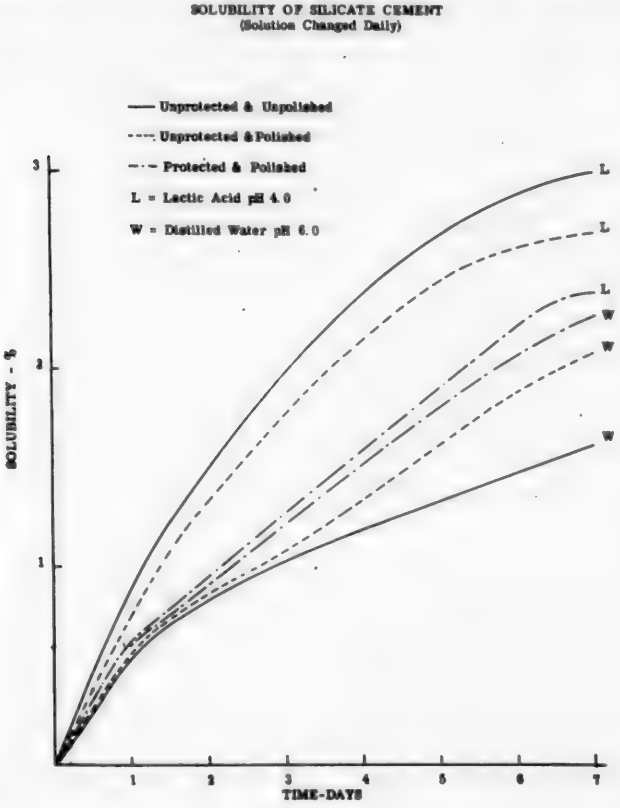

Fig. 3.

percentage difference between a high and a low powder-liquid ratio was only 1 per cent, this difference did represent a 100 per cent increase in solubility.

TABLE II

EFFECT OF POWDER-LIQUID RATIO ON SOLUBILITY OF SILICATE CEMENT

RATIO	MEDIA	
	CITRIC ACID pH 4.0	DISTILLED WATER pH 6.0
1.0 gram per 0.4 ml.	11.1%	2.0%
1.47 grams per 0.4 ml.	10.1%	1.0%
Standard deviation — 0.03%		

The manipulative variables did not seriously alter the rate of solubility of silicate cements in the media tested; however, other factors, such as strength and discoloration, are influenced by these procedures and adherence to these principles should not be discontinued on the basis of solubility studies alone.

Resins.—The results of the experiments where the commercial resins were stored in the same solution for periods of 24 hours and 1 week showed slight solubility (Table III). The data with distilled water corresponded to that reported by Schouboe.[2] In a few series, the salts of the acids were apparently sorbed from the solution and taken up by the resin discs. This was more

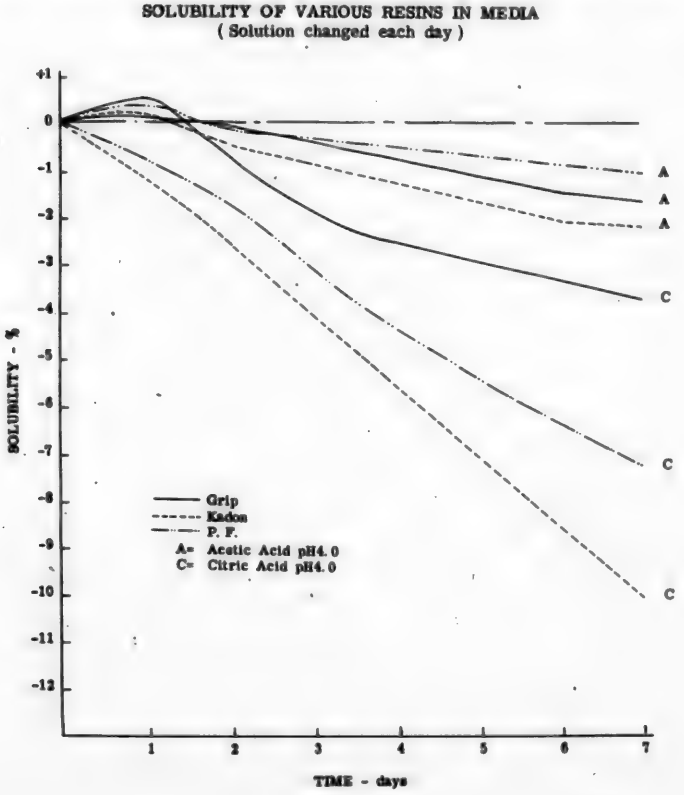

Fig. 4.

evident when the solutions were changed daily (Fig. 4). All specimens showed a weight gain. Likewise, in another series where the discs were stored for 10 days in the same solution and then the solution changed daily for 1 week, the same trend was noted (Fig. 5).

Cavity Liners.—The results with the cavity liners were similar to the results with the resins (Table IV). These liners are essentially natural gums, such as copal and rosin,[3] dissolved in an organic solvent. Discs of the varnishes were fabricated by a repeated application of the liners to the suspending

TABLE III
EFFECTS OF MEDIA AND pH ON THE SOLUBILITY OF RESINS

MEDIA	MATERIAL					
	GRIP (%)		KADON (%)		PF (%)	
	24 HR.	1 WEEK	24 HR.	1 WEEK	24 HR.	1 WEEK
Water, pH 6.0	0.39	0.06	0.27	0.03	0.20	0.36
Lactic acid, pH 4.0	0.28	3.40	–0.27*	0.41	0.61	0.43
Lactic acid, pH 5.0	0.58	4.69	2.20	1.06	0.55	1.84
Acetic acid, pH 4.0	0.39	0.46	0.52	0.16	0.35	0.58
Acetic acid, pH 5.0	–0.41*	0.74	0.61	0.68	0.15	0.66
Citric acid, pH 4.0	2.70	6.30	1.80	–0.74*	1.02	0.13
Citric acid, pH 5.0	0.57	1.13	0.20	0.03	0.65	–0.20*
Ammonium hydroxide, pH 8.0	0.43	0.49	0.20	0.03	0.26	0.70

Standard deviation — 0.12%

*Minus values indicate gain in weight of the discs.

SOLUBILITY OF VARIOUS RESINS IN CITRIC ACID pH 4.0
(Discs stored in the same solution for 10 days, then changed each day for 7 days).

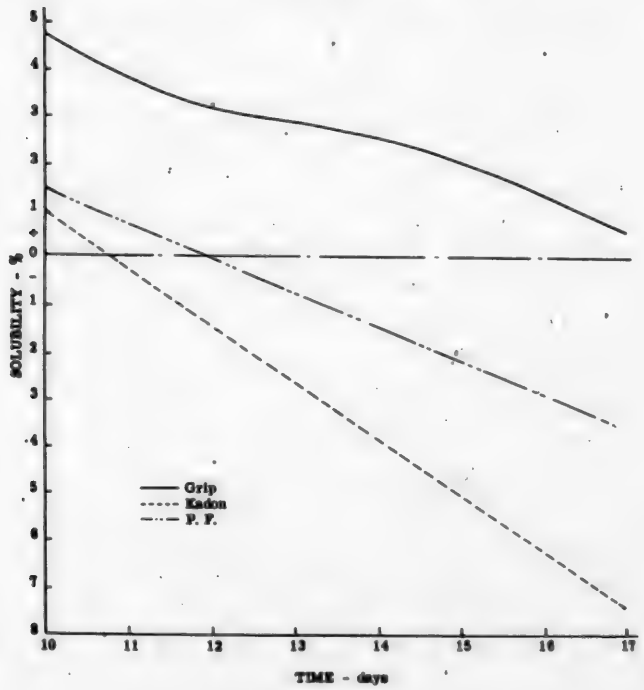

Fig. 5.

TABLE IV
PER CENT SOLUBILITY OF CAVITY LINERS AS EFFECTED BY CITRIC ACID

MEDIA	MATERIAL		
	COPALITE (%)	HANDI-LINER (%)	REPELAC (%)
Citric acid, pH 4.0	-1.38*	-1.35*	-6.13*

Standard deviation — 0.9%

*Minus values indicate gain in weight of the discs.

wires. The solvents were evaporated and applications were made until the discs were of adequate size.[1] The sorption by the cavity liners of the salts in the solution was, as could be expected, similar to that found for the resins.

Zinc Oxide and Eugenol.—There are little published data on the solubility of zinc oxide and eugenol. Some early clinical observations indicated that it is relatively insoluble,[4, 5, 6] and one laboratory study found the solubility in distilled water to be approximately 0.1 per cent.[7]

The solubility in several media can be seen in Table V. The zinc oxide-eugenol discs were quite soluble in the organic acids used. In one series, zinc acetate crystals (1 per cent) were added to the mix since clinically this is a common mode of accelerating the set. They had no appreciable affect on solubility. In previous research,[1] the gross effects of citric acid on the solubility of zinc phosphate and silicate cements were demonstrated. Here again, the solubility of zinc oxide and eugenol was greatest in this medium, the specimen completely dissolving in 1 week when the solution was changed daily.

TABLE V

SOLUBILITY OF ZINC OXIDE AND EUGENOL IN VARIOUS MEDIA IN SAME SOLUTION FOR 1 WEEK AND WITH SOLUTION CHANGED DAILY

	MATERIAL			
	WITH ZINC ACETATE CRYSTALS (%)		WITHOUT ZINC ACETATE CRYSTALS (%)	
MEDIA	1 WEEK	SOLUTION CHANGED DAILY FOR 7 DAYS	1 WEEK	SOLUTION CHANGED DAILY FOR 7 DAYS
	High Powder-Liquid Ratio			
Water, pH 6.0	2.55	0.94	0.89	0.74
Lactic acid, pH 4.0				1.66
Acetic acid, pH 4.0				4.41
Citric acid, pH 4.0	17.5	99.7	18.2	100.0
	Low Powder-Liquid Ratio			
Citric acid, pH 4.0	19.2			
	Standard deviation — 0.14%			

Since zinc oxide and eugenol mixtures are not uniform and vary considerably, the effect of powder-liquid ratio was studied. The data reported in Table V were obtained from specimens prepared with two powder-liquid ratios. It is conceivable that the solubility of zinc oxide and eugenol would be reduced by lowering the powder-liquid ratio. The eugenol is not markedly affected by aqueous solutions, being immiscible with water, whereas the zinc oxide is quite soluble in solutions of relatively low pH. The results of discs prepared with low powder-liquid ratio (only one half as much powder incorporated) and stored in citric acid, pH 4.0, showed much less dissolution over the same 7-day period. This resulted in a solubility of only 20 per cent as compared to the 100 per cent solubility in citric acid, pH 4.0, when a high powder-liquid ratio was employed.

Kryptex.—A commercial product (Kryptex), which is composed of both silicate and zinc oxide powders, was investigated. The effects of various media on the solubility of Kryptex can be seen in Fig. 6. Kryptex appeared to be less soluble in citric acid, pH 4.0, than either silicate or zinc phosphate cements. Otherwise, Kryptex cement behaved as one might anticipate, being generally less soluble than zinc phosphate cement and more soluble than silicate cement when the solutions were changed daily.

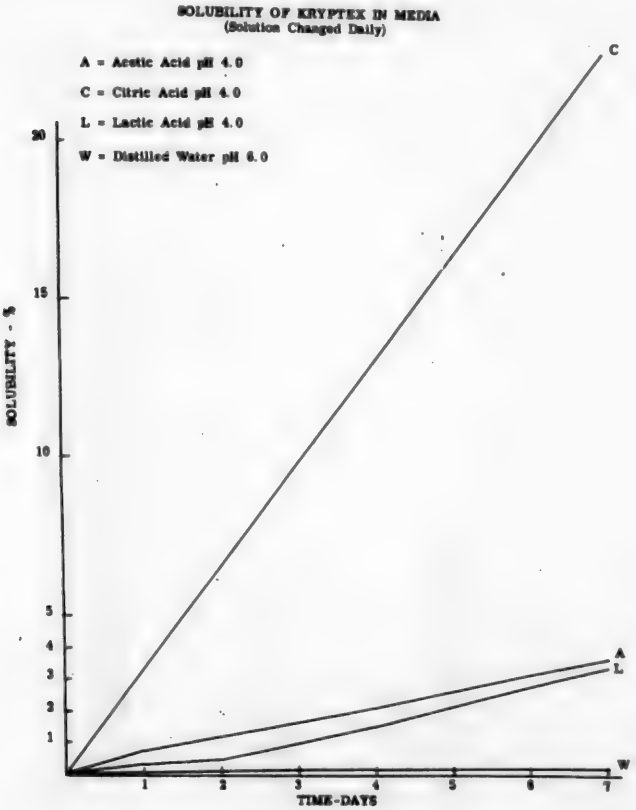

Fig. 6.

A summary can be seen in Fig. 7 which shows the deleterious effects of the various media upon zinc oxide and eugenol mixtures and Kryptex as compared with the data which had been previously obtained with zinc phosphate cement and silicate cement.

As pointed out in the first publication, the solubility of these materials is markedly influenced by the type and pH of the acids to which they may be exposed in the oral cavity. However, no attempt to correlate these data with the clinical behavior of the material should be made at this time.

SUMMARY

The solubility of resins, cavity liners, zinc oxide and eugenol, and Kryptex in various dilute organic acids and glass distilled water was investigated.

Silicate specimens continued to show marked dissolution in citric acid, pH 4.0, even after prolonged storage time. Certain manipulative variables did not markedly affect solubility of silicate cement. Generally, the resin materials

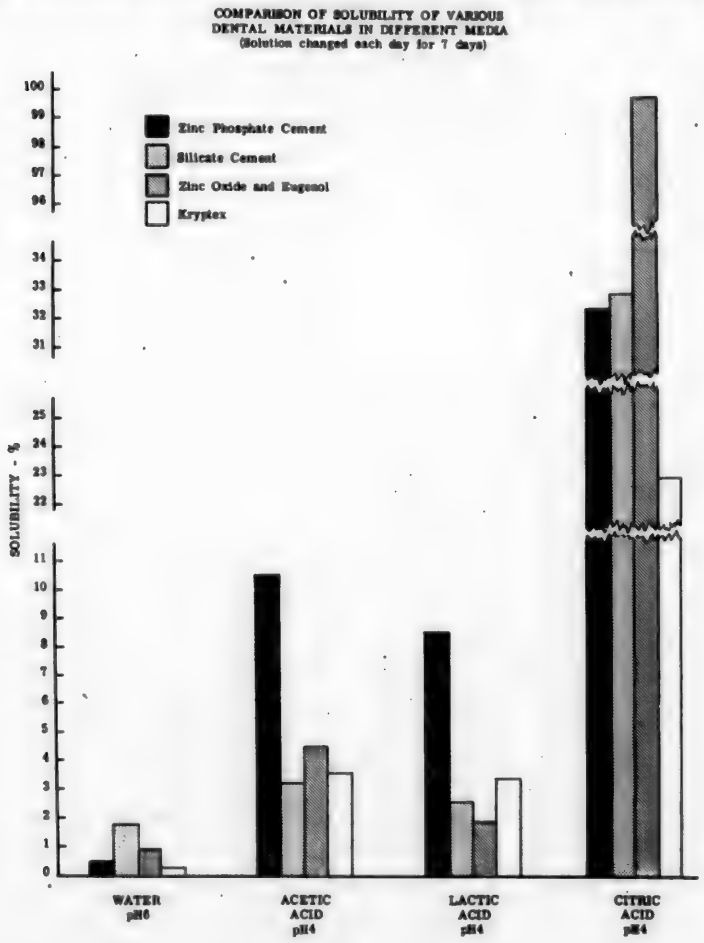

Fig. 7.

tested were relatively insoluble; when the acid solutions were changed daily, an actual weight gain occurred, presumably due to the sorption of salts from the solution. Citric acid again produced the greatest dissolution of the various materials. Mixtures of zinc oxide and eugenol were not quite as soluble in lactic acid or acetic acid as either zinc phosphate or silicate cements. Kryptex appears to be less soluble than zinc phosphate cement and more soluble than silicate cement.

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THE EFFECT OF CHRONIC VITAMIN A DEFICIENCY ON DENTAL CARIES IN THE SYRIAN HAMSTER

JOHN J. SALLEY, WILLIAM F. BRYSON,* AND J. ROBERT ESHLEMAN*

Department of Dental Research, Medical College of Virginia, School of Dentistry, Richmond, Va.

A RATHER well-accepted tenet among students of dental caries etiology is the inverse relationship which exists between decreased salivary secretion and caries incidence. Trimble, Etherington, and Losch[1] and Losch and Weisberger[2] have reported increased caries experience in human beings who demonstrated decreased salivary flow. These clinical observations have been substantiated in experimental animals by Weisberger, Nelson, and Boyle[3] and Cheyne[4] who noted increased caries scores in rats after surgical extirpation of major salivary glands. Similar observations have been made on desalivated hamsters by Gilda and Keyes[5] and Klapper and Volker.[6]

The development of a suitable synthetic diet for vitamin A deficiency studies in the hamster[7] has made it possible to investigate the effect of this dietary deficiency on dental caries activity. In this study a constant finding among A-deficient animals was squamous metaplasia of epithelial components in salivary glands, resulting in duct obstruction, sialadenitis, and suppurative degeneration of gland parenchyma.[7] These alterations of salivary gland structure closely agreed with findings in the rat reported by Wolbach and Howe.[8]

Because of salivary gland degeneration with avitaminosis A, it is not unreasonable to assume that some changes in oral environment may ensue. Indeed, the fact that both major and minor salivary glands are affected in the deficient animal[7] would lead to the predication that a situation somewhat analogous to gland extirpation may prevail during the deficient state. Shaw[9] has speculated on this question with reference to human caries mediated through xerostoma due, in turn, to the salivary gland alterations produced by inadequate consumption or assimilation of vitamin A.

In light of these corollary data and the fact that no direct experimental caries investigation has been done on vitamin A-deficient animals, it was felt that such a study should be conducted.

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*U. S. Public Health Service Student Research Fellow.

EXPERIMENTAL PROCEDURE

A total of 47 weanling albino hamsters* were divided into 2 littermated groups. Group I consisted of 32 animals and Group II of 25 animals. Both groups received the synthetic, vitamin A-free diet described in a previous report by Salley and Bryson[7]; however, Group II received 250 international units (I.U.) of vitamin A ester in Wesson oil (1,000 I.U./ml. solution) weekly. This ration had a carbohydrate fraction of 54 per cent in the form of powdered sucrose. Inasmuch as hamsters cannot survive without vitamin A for a length of time sufficient to conduct caries susceptibility experiments, animals in Group I were administered maintenance doses of 5 I.U. of vitamin A in Wesson oil (100 I.U./ml.) whenever necessary. Criteria for maintenance doses were continuous weight loss for 2 or 3 consecutive days, xerophthalmia, and fur changes. All administrations of vitamin A were given perorally. Animals were housed in wire cages with not more than 2 per cage. Individual weights were recorded daily and average group weights were plotted weekly.

The average time on the diet was 233 days for the depleted group and 366 days for the controls. At the time of death, livers were removed and analyzed for vitamin A content by the Carr-Price method. Maxillae and mandibles were also removed and placed in formalin prior to scoring for dental caries. After fixation, all soft tissues were stripped from the jaws, and they were examined under a dissection microscope (20× magnification) using a fine explorer. The method of scoring caries described by Keyes[10] was used. Histologic examination was performed on hematoxylin and eosin stained sections of major salivary glands, tongue, trachea, lungs, pancreas, kidney, and gonads.

RESULTS

Examination of the figures in Table I indicates that the deficient group had a total of three times more tooth structure destroyed by caries than the control group receiving the same ration but with adequate amounts of vitamin A added.

TABLE I

MEAN CARIES SCORES OF VITAMIN A DEFICIENT AND CONTROL HAMSTERS

	DEFICIENT				CONTROL			
	MA(%)	CN	AA	TS	MA(%)	CN	AA	TS
Maxillary	41.0	4.85	4.2	8.45	29.5	2.0	1.27	2.7
Mandibular	16.0	1.12	0.86	1.48	8.0	0.51	0.32	0.46
Totals	34.5	5.75	5.7	9.95	18.8	2.24	1.84	3.14

Legend: MA = molars affected; CN = number of carious lesions; AA = areas affected; and TS = total score.

Clinically, the molars of deficient animals showed more staining and debris on their coronal surfaces than did the controls. This was also a constant observation during the scoring procedure. In both groups the maxillary and mandibular second molars were the most caries-susceptible teeth in their respective arches; and it was also noted that the maxillary arch was more caries susceptible than the mandibular.

*Obtained from Pee Dee Farms, Driver, Virginia.

Fig. 1.

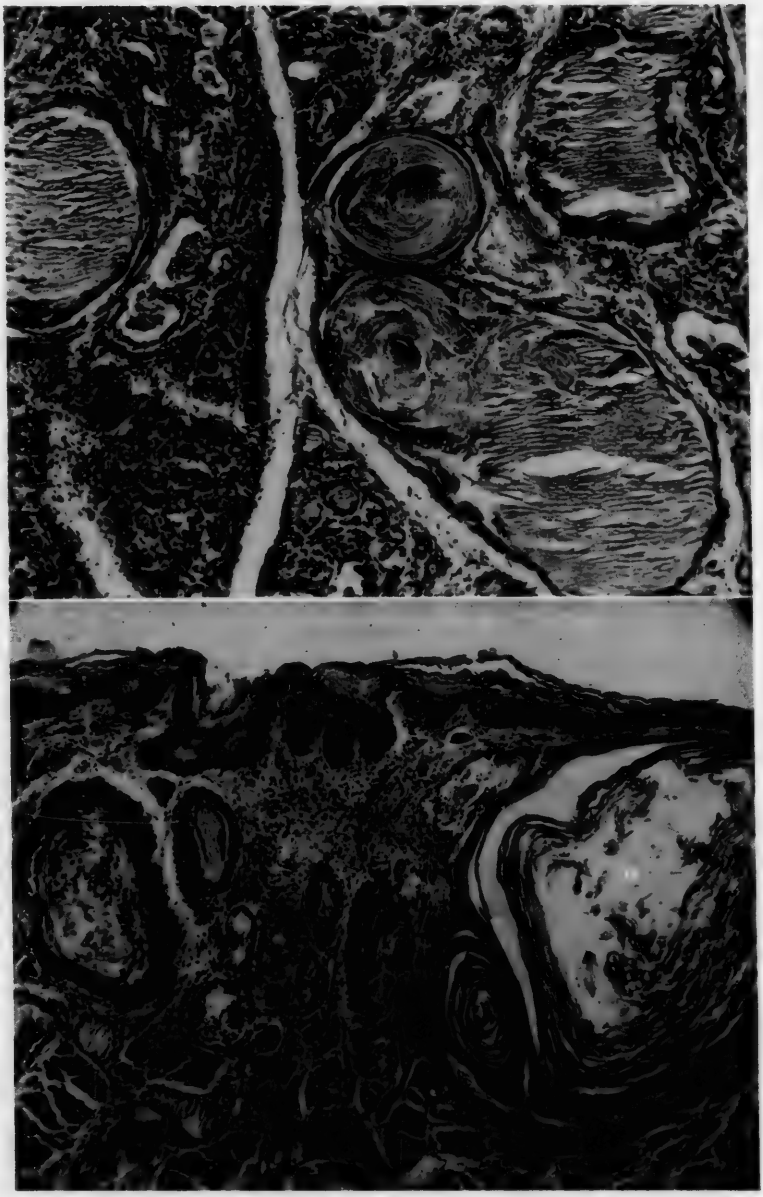

Fig. 2.

Fig. 1.—A section of parotid gland from a vitamin A-deficient hamster showing sialadenitis, squamous metaplasia of ductal epithelium, and several large keratotic plugs. (Hematoxylin and eosin; ×100.)

Fig. 2.—This photomicrograph is of the posterior portion of the dorsum of the tongue from a vitamin A-deficient animal. Minor salivary gland degeneration is illustrated in several mucous acini contained in the subepithelial connective tissue. Squamous metaplasia and keratotic plugging are the principal changes. (Hematoxylin and eosin; ×100.)

The histologic findings relative to vitamin A deficiency closely paralleled those reported for the hamster by Salley and Bryson.[7] The principal microscopic changes of interest in this study were in the salivary glands, both major and minor. Fig. 1 is a photomicrograph of the parotid gland of a hamster in Group I. Here one can see almost complete disappearance of secretory acini and replacement of gland parenchyma with infiltrates of acute and chronic inflammatory cells. Smaller ductal structures likewise are absent and the larger collecting ducts exhibit squamous metaplasia of ductal epithelium with large keratotic plugs. Other major glands showed similar changes. The glands of minor secretion were also affected in the deficient animals. An example of this finding is presented in Fig. 2 which illustrates replacement of mucous acini in the submucosal muscle layers of the posterior dorsum of the tongue

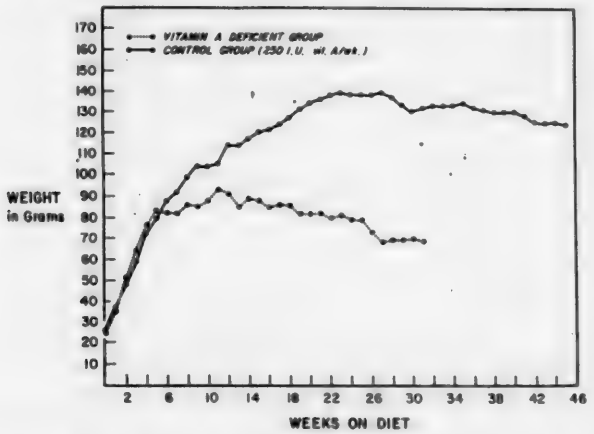

Fig. 3.—Composite weight curves for vitamin A-deficient and vitamin A-supplemented groups.

with keratin-filled plugs. These degenerative alterations in gland structure probably accounted for the rather marked xerostoma observed in the A-deficient group during the course of the study.

All other viscera examined histologically demonstrated the previously reported changes[7] compatible with vitamin A deficiency. Weight curves for the deficient and control groups are presented in Fig. 3. It can be seen here that a state of chronic deficiency resulted in a 37 per cent mean weight difference between the two groups. Results of liver analyses for residual vitamin A showed the depleted animals to have an average of only 10 I.U. of vitamin A per gram of liver, whereas control hamsters possessed a mean value of 275 I.U. per gram.

DISCUSSION

Since saliva plays an important role in the cleansing of teeth, salivary gland destruction resulting from chronic vitamin A deficiency offers the best explanation for the increased caries incidence in deficient animals. If such an assumption is valid, it may be inferred that the primary contributing factor of avitaminosis A to dental caries is one of a systemic effect on salivary gland

physiology. The question of developmental enamel defects[7, 8] due to A deficiency does not apply here since amelogenesis in the hamster molars was completed prior to the time of deficient manifestations.

A point of interest which the data in Table I raise is the relatively low caries scores in both groups in this experiment when compared to scores obtained by Dale, Lazansky, and Keyes.[11] These workers reported hamster caries incidence related to varying levels of fluoride intake. Since the method of scoring in their study was identical to that used in the present investigation, the scores are directly comparable. The mean score for two groups of hamsters ingesting minimal amounts of fluoride (0.4 ppm), as reported by Dale and his associates,[11] was 31.5 and 36.5. These figures indicate significantly more destruction of tooth structure than was seen in either the vitamin A-deficient group (9.95) or hamsters receiving supplements of vitamin A (3.14). In attempting to explain this discrepancy in scores, several factors should be considered. Aside from a possible difference in the cariogenic properties of the diets used in this and the other investigation, the question of fluoride intake must be examined. The synthetic ration consumed by both A-deficient and control groups contained 9 ppm of fluoride as NaF. Additionally, the drinking water to both groups contained 1 ppm NaF for a total of 10 ppm. Two groups of hamsters in the study by Dale and his co-workers[11] received 50 and 68.3 ppm fluoride with average caries scores of 5.8 and 5.2, respectively. It is possible that the 10 ppm ingested by our animals afforded some degree of caries resistance, but not in any way proportional to that achieved with 50 to 60 ppm.

Probably one of the more important factors is strain difference. It is possible that our hamsters may represent a more caries-resistant strain than those used by Dale.[11] The problem of hereditary factors in hamster caries has been outlined by Johansen and Hodge.[12] It is entirely possible that strain difference is paramount in this comparison especially when one compares the results of these two studies on the basis of experimental period. Dale and his associates indicated an experimental period of only 125 days, whereas our deficient hamsters were on the diet for 233 days, with controls extending to 366 days. The difference in experimental period between the control and A-deficient animals was not desirable, but resulted from earlier mortality caused in turn by the deficient state. However, it is the ratio of the average deficient score to the average control score (3.2:1) which we feel is significant. Had the experimental periods for both groups in this study been of equal length, this ratio may have been even greater.

One more finding of note concerns the comparative susceptibility of the maxillary and mandibular molars. Although Keyes and Shouri[13] showed that hamster maxillary molars are slightly more caries susceptible than are the mandibular molars, it is difficult to explain why the ratio of maxillary to mandibular total score (5.7:1) was so high in this experiment. This ratio was almost the same for both groups. The fact that the mandibular teeth were bathed in saliva (even though reduced) to a greater extent than were the maxillary teeth might possibly have been a factor.

SUMMARY

A total of 47 weanling hamsters was divided into two groups. Both groups were fed a synthetic diet containing all nutritional requirements except vitamin A. The control group received 250 I.U. of vitamin A weekly and the deficient group was given 5 I.U. of vitamin A whenever necessary to maintain life. At autopsy, the jaws were removed and carious lesions scored by a volumetric method. Results showed that the deficient animals had over three times as much tooth structure destroyed as the controls. Reduced salivary flow due to the effects of vitamin A deficiency on major and minor salivary glands probably accounted for increased susceptibility to caries.

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THE ORAL YEAST-LACTOBACILLUS RELATIONSHIP

II. Yeast-Lactobacillus-Saliva Interrelationships

THOMAS E. WILSON* AND PAUL W. GOAZ

Dental and Microbiological Research Section, Oklahoma Medical Research Foundation, Oklahoma City, Okla.

IN SPITE of the fact that the lactobacillus has received the greatest amount of attention, its status as the etiological agent of dental caries is still in doubt. However, the active role played by oral bacteria in the acid decalcification of tooth substance is reasonably well accepted as one of the initial steps in the pathogenesis of decay. Therefore, any factor that contributes to the acidogenic capacity of the lactobacillus or any prominent oral microorganism assumes importance in the interpretation of the factors contributing to the development of the carious lesion.

Such a contributory factor was demonstrated by the work of Fosdick and his group[1] when it was shown that a mixed culture of oral yeast and lactobacilli apparently produced larger quantities of acid during a specific period of incubation than could be attributed to the sum of the activities of the individual microorganisms in pure culture. Subsequent studies with dried cells[2, 3] indicated that rapid lactate production by each of these microorganisms was precluded by different rate-limiting steps. The inability of the yeast to rapidly reduce pyruvate and the limited capacity of the lactobacillus to phosphorylate glucose appeared to be compensated by a relatively greater rate of these activities in the companion organism. These findings led to the suggestion that the enhanced activity of the mixed culture could be explained on the basis of a complementary effect of the glycolytic enzymes from each. However, the explanation was dependent upon the speculation that the phosphorylating enzyme system of the yeast, or its products, is available to, or utilizable by, the lactobacillus. This suggestion does not seem very probable, since it has been demonstrated that the phosphorylated hexose intermediates of glycolysis are not usually actively transported into intact cells,[4] and that a lactobacillus did not utilize them for growth or acid production.[5]

Part of these data were presented at the Thirty-seventh Annual Meeting of the International Association for Dental Research, San Francisco, Calif., March 21, 1959.

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*Present address: Department of Bacteriology, Southwestern Louisiana Institute, Lafayette, La.

Subsequently, it was shown by Challinor and Rose,[6] who used nonoral strains for their study, that this interrelationship was apparently of a syntrophic nature with yeast supplying the lactobacillus some thirteen growth factors and essential metabolites. Two years later, Young and her co-workers[7] demonstrated with oral microorganisms that a candida supplied a lactobacillus with four of its required B vitamins, and this work was recently confirmed and extended by Koser[8] with a number of oral strains of lactic acid bacteria.

Insofar as the investigations, to date, emphasize the fact that yeast apparently supplies the lactobacillus with certain essential nutrient factors that stimulate its growth and acid production, it seemed of interest to determine how important this role of the yeast would be under conditions which more closely approximated the in vivo environment, i.e., in the presence of autoclaved saliva. Since saliva has been reported to contain some of the nutritional factors supplied by yeast,[9-12] the growth of an oral lactobacillus in a basal medium, fortified with saliva, has been compared with its growth in the medium preconditioned by the growth of a *Candida albicans*, and is the subject of this report.

METHODS

Medium.—A partially defined medium was employed for the vitamin assays, and had the following composition per liter: 6.0 Gm. sodium acetate, 5.0 Gm. casein hydrolysate, 10 mg. L-tryptophane, 10 mg. L-cysteine, 5 mg. L-asparagine, 10 mg. adenine, 10 mg. guanine, 10 mg. uracil, 10 mg. xanthine, 250 μg thiamine, 250 μg niacin, 250 μg calcium pantothenate, 50 μg pyridoxamine, 250 μg riboflavin, 5 μg biotin, 2 μg folic acid, 1.0 Gm. K_2HPO_4, 1.0 Gm. KH_2PO_4, 125 mg. $MgSO_4$, 10 mg. $FeSO_4$, 10 mg. NaCl, and 20 Gm. of glucose (autoclaved separately).

A completely synthetic basal medium was used for the amino acid determinations and corresponded to the vitamin assay medium, except for the replacement of the casein hydrolysate and amino acid supplement by a mixture of 18 amino acids, each at a concentration of 100 μg per milliliter.

Cultures.—An oral strain of *Lactobacillus casei*, obtained from the Zoller Dental Clinic, University of Chicago, and a *Candida albicans* isolated from human saliva were used as test microorganisms. Stocks of both were maintained on TJY agar slopes stored at 4° C. The inoculum of either microorganism was prepared by washing a 20- to 24-hour culture, grown in the partially defined medium containing 0.1 per cent glucose, and resuspending to original volume in distilled water. Each 10 ml. of test medium received 0.001 ml. of the resulting suspension of the appropriate microorganism.

The observation that a cellophane barrier did not prevent the growth of *L. arabinosus* and *Streptococcus faecalis* R in an association that required an exchange of phenylalanine and folic acid,[13] led to the adaption of a diphasic culture system for investigation of the capacity of an oral *C. albicans* to supply essential nutrients for the lactobacillus (Fig. 1). This diphasic system was contrived by growing the candida within a viscose bag (inner phase) that was

suspended in a flask of the assay broth (outer phase). The viscose bag contained approximately 10 per cent of the total volume of assay medium in the complete system, and was so arranged that it could be withdrawn from the (yeast conditioned) outer phase after any suitable period of incubation. The medium in the outer phase could, after inoculation with the *L. casei*, and appropriate conditioning by the yeast, be transferred aseptically to sterile tubes for further incubation and turbidimetric measurement of growth. This system obviates the difficulty of quantitating results from mixed colonies and eliminates the relatively cumbersome plating technic for measuring growth through the use of the more convenient turbidimetric methods. The period the medium was exposed to the candida was controlled in order to restrict the nutrient factors detected to those which would be released by actively growing cells, and to avoid, as much as possible, the contamination of the system by those compounds derived from older autolyzing yeast cells.

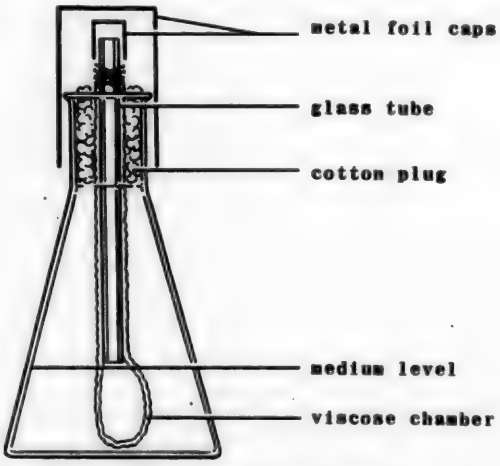

Fig. 1.—Diphasic culture system.

In evaluating the results obtained from such a diphasic culture system, at least two characteristics of the technic should be considered. Primarily, this method permits the detection of only those compounds that are capable of diffusing through the viscose membrane. This necessarily precludes the response of the test microorganism to the protein-bound, or large molecular weight, materials which might otherwise be utilized. In addition, it is apparent that a concentration factor reduces the sensitivity of the system. The microorganism within the inner phase must produce the growth-promoting material to be demonstrated, at a concentration that exceeds the effective level at which the test microorganism in the outer phase responds. This sensitivity factor is imposed by the ratio of the volumes of the outer to the inner phase of the culture system. This is in contrast to conditions in a mixed culture per se, where the recipient microorganism often grows in proximity, or even adheres, to the donor strain.[7] The resulting spatial relationship permits the utilization of materials at the site of their release and minimizes any dilution factor.

RESULTS AND DISCUSSION

The relatively more rapid growth of the yeast, coupled with its apparent syntrophic role, seemed to relieve the necessity of inoculating both phases of the culture system at the same time. This was confirmed by demonstrating that the maximum growth of the lactobacillus in a complete, but limiting, medium was not significantly different when both phases of the culture system were simultaneously inoculated, or when the medium was preconditioned for 20 hours by the growing yeast (Table I). Also, when this comparison was made in the vitamin assay medium, there was little difference in the growth recorded in the simultaneous and preconditioned systems. Inasmuch as the mechanics of handling the preconditioned system were less rigorous, this technic was used throughout the study.

TABLE I
COMPARISON OF PRECONDITIONED MEDIUM AND SIMULTANEOUS CULTURE TECHNICS

VITAMIN OMITTED	CONTROL*	SIMULTANEOUS CULTURE	PRECONDITIONED MEDIUM
None	0.383†	0.399	0.382
All	0.001	0.007	0.005
Pyridoxamine	0.007	0.401	0.415
Riboflavin	0.005	0.252	0.228
Ca pantothenate	0.022	0.167	0.175
Folic acid	0.028	0.032	0.017

*Not exposed to *C. albicans*.
†Optical density—maximum.

The usual inference, drawn from the many in vitro studies of growth and acid production by oral lactobacilli, is that the imposed circumstances and results are probably reasonably applicable to the complex, in vivo environment. Although, it has been demonstrated that candida will promote better growth of lactobacilli by supplying required vitamins and amino acids,[6, 7, 8] a consideration of the complement of these same factors in saliva suggested that the relative value of the yeast-produced factors may be of completely different magnitude in the oral environment. While the concentration of vitamins and amino acids may vary markedly from sample to sample of saliva, or even in samples from the same individual collected at different times, all the essential B vitamins and amino acids might be expected to be present in effective amounts in an aliquot from a pooled sample of saliva.[9-12] Accordingly, some experiments were conducted employing medium supplemented with 25 per cent autoclaved, pooled, whole saliva.

Preconditioning by the yeast of the unsupplemented, vitamin-deficient medium promoted reasonably good growth of the lactobacillus when any one of the essential vitamins, except folic acid, was omitted. In comparison to the growth obtained in the complete control medium, growth in the preconditioned assay medium ranged from a low of 7 to 8 per cent in the absence of folic acid (only 2 per cent over the growth recorded in the medium from which folic acid was omitted) to a high of 130 per cent when pyridoxamine was omitted (Fig. 2). Other growth response comparisons of the vitamin activities supplied by the

yeast, to that in the complete medium, were 50 per cent for niacin, 45 per cent for calcium pantothenate, and 60 per cent for riboflavin. The biotin and thiamine levels in the casamino acid component of the medium were of sufficient magnitude to preclude the demonstration of these requirements. The data for biotin, however, are included for comparison and, in effect, constitute another control. The slight growth observed in the absence of the essential vitamins is assumed to reflect the presence of traces of these, or closely related compounds, in the components of the medium..

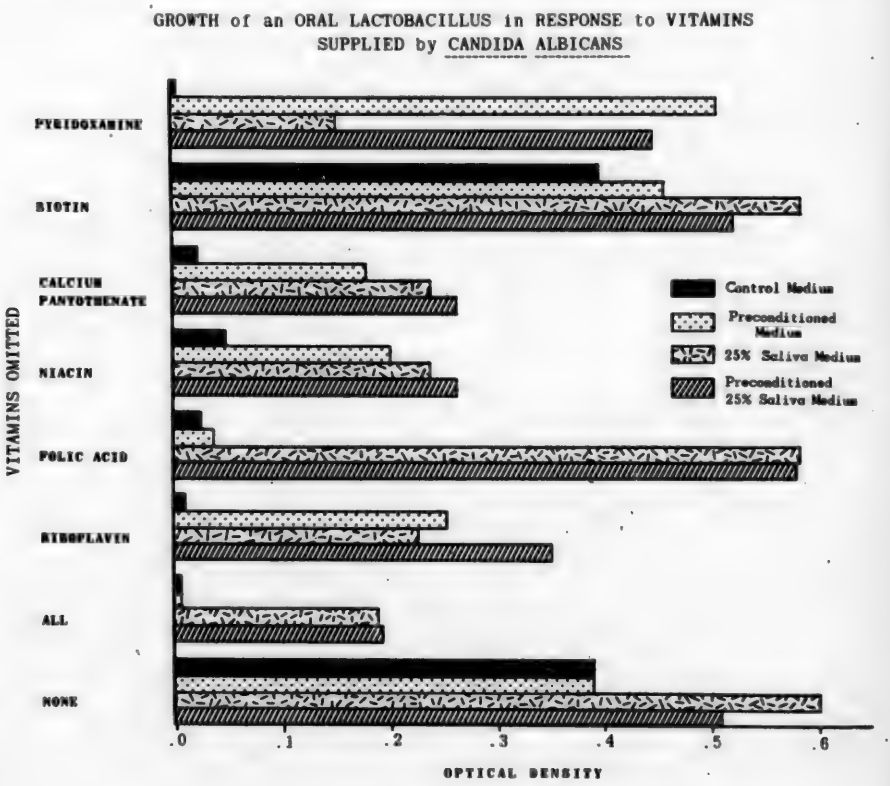

Fig. 2.

To evaluate the importance of these growth factors actively secreted by the yeast, the vitamin assay medium was supplemented with 25 per cent saliva. It was observed that this level of saliva promoted from moderate to good growth of the lactobacillus, notwithstanding the serial omission of the individual essential vitamins (Fig. 2). Particularly striking was the effectiveness of saliva in replacing folic acid activity, causing a response that was approximately twofold that attained when any other essential vitamin was omitted from the medium. In general, saliva contained all the vitamins initially shown to be essential for the test microorganism. However, it is apparent that some of the vitamins, or vitamin-like compounds, are present at limiting concentrations as indicated by

a restricted but moderate growth, in the saliva-supplemented medium from which all the six vitamins (except those inadvertently present) were withheld. In addition, growth in the complete medium was greater in every case than when a vitamin was replaced by saliva, except in the case of folic acid. However, in comparison to the syntrophic effect of the yeast, it is to be noted that the response to saliva was, in the case of every vitamin omitted, except pyridoxamine, equal to, or greater than, that effected by the yeast. Also, the response to the saliva-supplemented medium, when all the vitamins were omitted, was about 50 per cent of that in the complete medium, while the corresponding response in preconditioned medium was only 5 per cent or less.

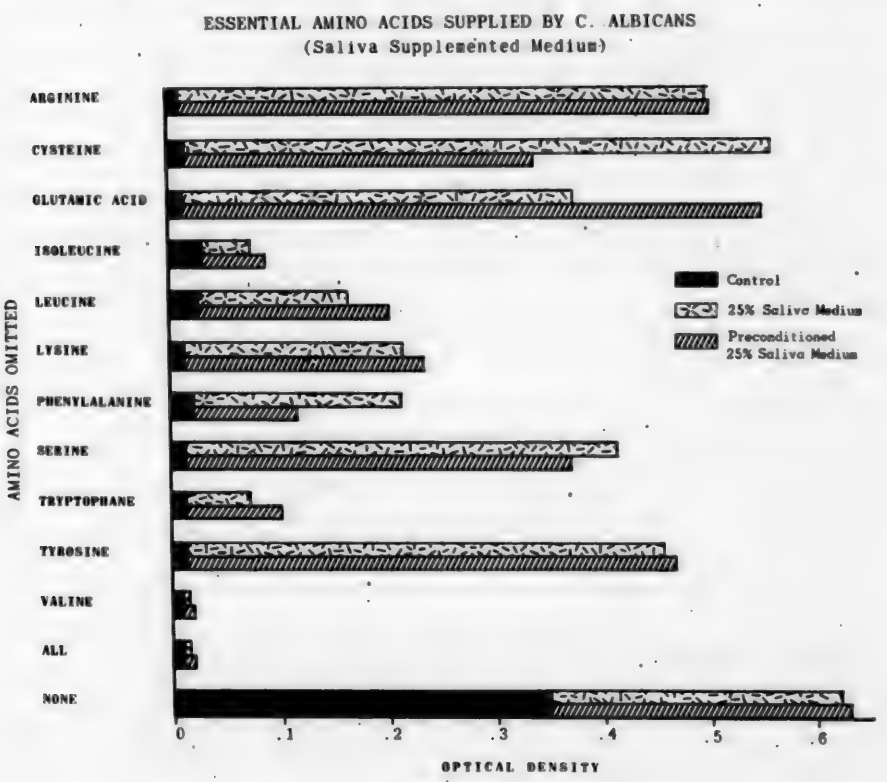

Fig. 3.

On the basis of single omission tests, arginine, cysteine, glutamic acid, isoleucine, leucine, lysine, phenylalanine, serine, tryptophane, tyrosine, and valine were shown to be essential for the growth of the test lactobacillus in the completely synthetic medium containing the remaining ten essential, and an additional seven nonessential, amino acids. These omission tests were then repeated to demonstrate the degree of amino acid replacement that would result from preconditioning the same medium with the yeast, fortifying it with 25 per cent saliva or a combination of both procedures.

Preconditioning the synthetic medium for either 20 or 46 hours did not result in any detectable change in the concentration of the essential amino acids, except lysine, which was only apparent after the 46-hour preconditioning period (Table II). In contrast, saliva at the 25 per cent level was shown to be a good source of five of these essential amino acids and a fair source of three others (allowing about one third of the maximum growth) (Fig. 3). Only in the absence of isoleucine, tryptophane, and valine, is growth severely limited in the saliva-fortified medium. Preconditioning of the saliva-supplemented medium did not result in an increased supply of these three. The yeast growing in the saliva-supplemented medium effectively increased the level of glutamic acid, although, the significance of this observation would seem to be limited, in view of the fact that it is one of the amino acids saliva provides at a reasonably effective concentration. In the preconditioned, saliva-supplemented medium, reductions in the amounts of cysteine and phenylalanine available to the lactobacillus were apparent, indicating a utilization or destruction of these by the candida.

TABLE II

ESSENTIAL AMINO ACIDS RELEASED BY C. ALBICANS IN A SYNTHETIC MEDIUM

ESSENTIAL AMINO ACID OMITTED	CONTROL MEDIUM*	PRECONDITIONED MEDIUM	
		20 HOURS	46 HOURS
Arginine	0.009†	0.018	0.020
Cysteine	0.055	0.008	0.090
Glutamic acid	0.010	0.008	0.000
Isoleucine	0.011	0.022	0.005
Leucine	0.041	0.060	0.020
Lysine	0.006	0.010	0.255
Phenylalanine	0.017	0.010	0.006
Serine	0.005	0.009	0.023
Tryptophane	0.011	0.040	0.011
Tyrosine	0.013	0.024	0.013
Valine	0.012	0.026	0.001
All	0.007	0.008	0.000
None	0.348	0.306	0.290

*Not exposed to *C. albicans*.
†Optical density—maximum.

Under the conditions employed, the candida does not supply sufficient amounts of the essential amino acids to supplement those which are available in saliva, or to have a significant effect upon the growth of the lactobacillus. Any stimulatory effect which the candida might exert upon the lactobacillus would, therefore, seem to exclude the amino acids as an active principle. By contrast, the candida was an excellent source of several vitamins. However, the value of the candida as a source of vitamins was minimized in the presence of saliva. Only in those cases where a saliva sample might have a very low concentration of one or more vitamins would it seem reasonable to assume that the candida would have a significant effect upon the growth or acid production of the lactobacillus.

The demonstration of a specific syntrophic activity requires the use of an appropriately deficient medium. This activity can be rendered ineffective, or

at least inapparent, when there is a plentiful supply of the particular factor involved. Consequently, an investigation which seeks to elucidate the significance of any syntrophic activity, with relation to the natural environment, must take into consideration the natural supply of the factor under investigation.

SUMMARY

In a diphasic culture system, an oral strain of *Candida albicans* is an active donor of materials with pyridoxamine, calcium pantothenate, niacin, and riboflavin activity. The *C. albicans* did not supply a significant amount of folic acid, or any essential amino acid, except lysine. Pooled, whole saliva contained all of the vitamins, and eight of the eleven amino acids essential for the growth of the lactobacillus. In the presence of saliva, the syntrophic effect of the yeast, attributed to the release of vitamins, was minimized, due to the availability of these same factors in the saliva.

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RESEARCH ANNOTATIONS*

THE EFFECT OF YEAST ON DENTAL CARIES IN THE RAT

WOLFGANG BUTTNER** AND JOSEPH C. MUHLER

Department of Biochemistry, Indiana University Medical Center, Indiana University, Indianapolis, Ind.

IN A PREVIOUS study of the effect of fish meal on the incidence of dental caries in the rat, one group of animals received 10 per cent yeast (Buttner, W., and Muhler, J. C.: *J. D. Res.* 37: 419, 1958). In these latter animals there was a marked increase in the incidence of dental caries as well as a significant failure of the animals to gain weight normally. Since yeast is considered by many to be nutritionally beneficial, it seems of importance to learn more about the nature of this cariogenic effect.

Experimental.—A total of 200 weanling Sprague-Dawley strain rats were divided into 5 groups according to sex and initial body weight. Group 1 consisted of animals which received a stock corn diet (SCD) composed of (in per cent): yellow corn grits, 52.7; ground whole yellow corn, 11.3; powdered whole milk, 30.0; alfalfa, 4.8; iodized sodium chloride, 1.0; and irradiated yeast, 0.2. The animals in Groups II and III received the same diet as those in Group I except that 3 and 6 per cent, respectively, of the ash of this diet was added at the expense of equal amounts of corn grits and powdered whole milk. The animals in Group V received the same diet as those in Group IV except that 10 per cent yeast ash (by volume) was added instead of irradiated yeast. All of the diets, along with fluoride-free water, were available ad libitum. The animals were housed in pairs in raised screen cages in an air-conditioned room for 120 days, were sacrificed and the heads examined for dental caries (Muhler, J. C., Nebergall, W. H., and Day, H. G.: *J. D. Res.* 33: 33, 1954).

Data and Discussion.—The animals receiving 6 per cent SCD-ash failed to gain as much weight as the controls (Table I), and, while a similar tendency was evident in the animals receiving the SCD-ash at the 3 per cent level, the results were not nearly as pronounced. The most pronounced failure to gain weight was noted in the animals receiving the 10 per cent yeast. During the experimental period the males gained about 100 grams less and the females about 60 grams less than their respective controls. When the yeast ash was added, no differences in weight gain were noted.

The incidence of dental caries in the animals which received the SCD-ash was not significantly different from the controls.

There was a significant difference ($p = 0.02$) between the weight gain of the animals receiving the SCD-ash at the 6 per cent level when compared to the controls, but no difference when 10 per cent yeast ash is added. Of considerable interest is the group receiving 10 per cent irradiated yeast ash. Apparently the yeast contains a heat-liable component which is destroyed upon ashing and, when absent, fails to inhibit growth of the animals. These effects on weight are similar to those previously reported (Buttner, W., and Muhler, J. C.: *J. D. Res.* 37: 419, 1958).

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**Postdoctorate Fellow. Present address is the University of Mainz, Mainz, Germany.

TABLE I. THE EFFECT OF YEAST ON THE INCIDENCE OF DENTAL CARIES IN THE RAT

GROUP	SEX	NO. OF RATS	MEAN WEIGHT GAIN (GM.)	MEAN NO. OF LESIONS (SEXES COMBINED)	P*
I	♂	18	215	7.9 ± 0.4†	—
	♀	15	135		
II	♂	12	189	6.6 ± 0.6	0.10
	♀	16	121		
III	♂	12	143	7.1 ± 0.5	0.20
	♀	16	108		
IV	♂	13	118	9.5 ± 0.6	0.05
	♀	17	75		
V	♂	15	205	6.4 ± 0.4	0.04
	♀	14	120		

I = SCD; II = 3% Stock Corn Diet (SCD) ash; III = 6% SCD-ash; IV = 10% yeast; V = 10% yeast ash.
*Calculated on the basis of the "t" test.
†Standard deviation.

When irradiated yeast was added to the diet, a significant increase in the incidence of dental caries occurred. However, when irradiated yeast was ashed and the ash added to the diet the cariogenic effect was eliminated. Instead, this diet had a significant anticariogenic effect. Apparently the irradiated yeast contains a heat-labile component which not only affects body weight but also is related in some systemic manner to the incidence of dental caries. This factor(s) is not known, but its recognition may be of real importance in our basic understanding of the caries process and means of prevention of human dental caries.

Summary.—The addition of 10 per cent irradiated yeast to a stock corn diet significantly increased its cariogenic effect, as well as significantly inhibiting the growth of rats. When the yeast was ashed and then added to the diet, both the cariogenic and the growth-inhibiting effects were lost. The addition of ash obtained from the stock corn diet had no effect on the dental caries experience. Apparently, irradiated yeast contains an unrecognized heat-liable factor(s) which not only inhibits body growth but also acts as a cariogenic agent.

THE EFFECT OF THE TOPICAL APPLICATION OF STANNOUS FLUORIDE ON ENAMEL SOLUBILITY WHEN PRETREATED WITH SODIUM FLUORIDE

JOSEPH C. MUHLER AND WOLFGANG BUTTNER*

Department of Biochemistry, Indiana University, Indiana University Medical Center, Indianapolis, Ind.

THE REACTION between enamel and stannous fluoride has been shown to produce a product highly insoluble in acid. While the exact chemical nature of this product is not known, it is thought to consist of a combination of tin and phosphate, tin and oxygen, or a combination of both. During electron microscope and electron defraction studies using whole teeth, it was found that, while the enamel reaction product was extremely acid resistant, it was located only throughout the outermost enamel layers. If this protection is lost by mastication, toothbrushing, and the like, the acid resistance of the enamel may be lost, or significantly decreased. Since chemical analysis of the various enamel layers has indicated that the depth of penetration of sodium fluoride is greater than that of stannous fluoride, due undoubtedly to the pronounced affinity of the stannous ion with phosphate, which in a

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*Post-Doctorate Fellow. Present address is the University of Mainz, Mainz, Germany.

sense, limits its depth of penetration, it became of interest to learn what effect the pretreatment of enamel with sodium fluoride would have on the effectiveness of a single application of stannous fluoride.

A total of 36 young adult caries-free rats were sacrificed and their mandibular molars prepared for determinations of acid solubility by methods previously described (J. D. Res. 36: 897, 1957). These embedded molars were divided into 6 groups as shown in Table I,

TABLE I. THE EFFECT OF COMBINED PROTECTIVE TREATMENT WITH SODIUM FLUORIDE AND STANNOUS FLUORIDE ON THE IN VITRO ENAMEL SOLUBILITY OF RAT MOLAR TEETH (6 HEMIJAWS PER GROUP)

			MEAN μg PHOSPHORUS DISSOLVED (IN SEPARATE 10 MIN. ACID TREATMENTS)											
			1		2		3		4		5		6	
	PROTECTIVE TREATMENT			%		%		%		%		%		%
GROUP	FIRST	SECOND	μg P	RED.	μg P	RED.	μg P	RED.	μg P	RED.	μg P	RED.	μg P	RED.
1	Distilled water	—	70-3‡	—	62-2	—	71-1	—	55-4	—	66-2	—	56-3	—
2	NaF*	—	31-3	55	34-3	45	33-4	54	34-3	38	40-3	39	33-2	41
3	SnF_2†	—	14-1	80	20-2	68	29-3	59	28-2	49	33-2	50	35-3	38
4	NaF	NaF	29-3	58	25-1	60	26-2	63	29-2	47	27-3	59	27-3	52
5	SnF_2	SnF_2	8-2	89	16-3	74	23-4	68	28-4	49	32-3	51	34-3	39
6	NaF	SnF_2	9-1	87	14-1	77	19-1	73	21-1	62	23-2	65	23-2	57

*4 per cent, pH 7.5.
†8 per cent, pH 2.8.
‡Standard deviation.

and estimations of their acid solubility determined following protection with either sodium or stannous fluoride. The 2nd, 3rd, 4th, 5th, and 6th decalcifications were conducted using new acetate solution for each subsequent decalcification.

The group treated with distilled water indicated a decreasing loss of phosphate as a function of time (Table I). The first acid treatment removed 70 μg phosphate and the sixth, 56 μg. This reduction in phosphate loss must indicate either a lessened solubility of the enamel layers further removed from the surface, or the presence of some acid-inhibiting factors formed on the enamel surface which prevented subsequent acid dissolution.

When the intact molars were treated with 4 per cent sodium fluoride and with acetate, 31 μg phosphate was removed, and a protection against acid decalcification of 55 per cent resulted. Approximately this same amount of phosphate was removed each time the enamel was treated with new acetate solution. It would appear from these data that the sodium fluoride with which the teeth were treated penetrated deep into the enamel, thus preventing the decreased rate of phosphate removed as observed in the group treated with distilled water.

The intact molars treated with an 8 per cent solution of stannous fluoride had considerably more protection initially from acid dissolution than similar teeth treated with sodium fluoride. However, this increased protective effect of stannous fluoride was promptly lost, since after three separate acid treatments the stannous fluoride was no more effective than the sodium fluoride.

Two treatments of sodium fluoride did not appear to be more effective than one treatment and, initially, the combination was not as effective as one treatment of stannous fluoride. However, two applications of stannous fluoride were more effective than either two of sodium or stannous fluoride initially. This protective effect was also lost, but not as quickly as the effect from the single application.

The teeth treated in Group 6, which received a single application of sodium fluoride, followed immediately by a single application of stannous fluoride, not only had a low initial solubility (9 μg phosphate removed), but the duration of effectiveness was superior to any other treatment or combination of treatments.

These data would suggest that a combination of a single application of sodium fluoride and stannous fluoride would be superior to either one alone, but, clinical tests must be conducted in order to test this hypothesis.

ACID PRODUCTION AND THE CARIOUS PROCESS

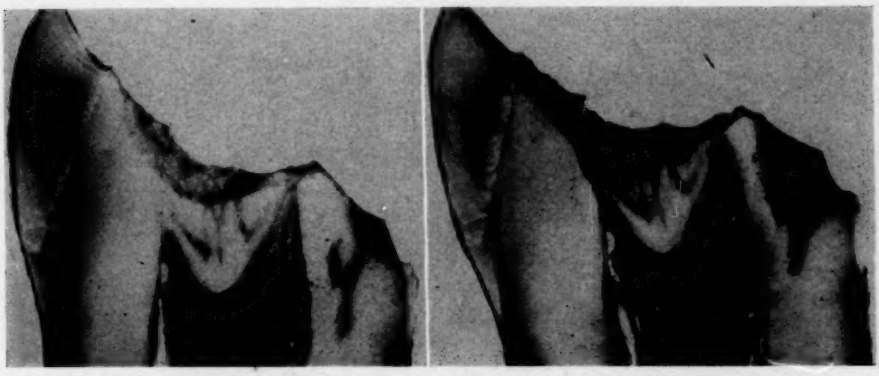
ALEXANDER B. MAC GREGOR

The University of Birmingham, Birmingham, England

THE ASSOCIATION of acid production with the carious process has been generally accepted for some time, although evidence of the actual presence is scanty. It has also been suggested that the carious process in the enamel might be different from that in the dentine and, whereas acid might be present in the former, proteolytic destruction of the dentine might not be associated with the presence of acid.

A simple technic has been devised which demonstrates the presence of acid in carious enamel and dentine and gives some indication of the relative amounts of acid in different zones.

Technic.—The crown of the freshly extracted carious tooth was cut off, and multiple sections, each about 200 μ thick, were cut through the crown with a diamond wheel cooled with distilled water. The sections were removed and mounted on a glass slide and a few drops of sodium methyl red (aqueous acid methyl red neutralized with caustic soda, pH range 4.4-6.0) were placed on the sections. An immediate color reaction was noted, the altered and carious



A. *B.*

Fig. 1.—A badly carious tooth after removal of most of the softened dentine. *A*, Before application of methyl red indicator. *B*, After application. The dark areas of dentine seen in *B* are bright red while the rest of the dentine is yellow.

enamel turning bright pink and the carious dentine also turning red while the rest of the enamel and dentine showed a yellowish coloration. It is of considerable interest that even small and early patches of altered enamel frequently showed a bright red coloration as did the dentine, not only in the discolored grossly carious area but particularly at the advancing edge of the carious process. Numerous indicators were tried, but many, e.g., bromcresol purple, bromcresol green, and chlorphenol red, suffered from the defect of turning to yellow on the acid side. This was not so easy to distinguish against the background of the tooth as the bright red reaction to acid of the methyl red. Enough sections have been done with other indicators, however, to show that the areas showing an acid reaction are common to all the indicators. In the case of chlorphenol red, the pH of altered enamel and dentine was below 5.5, and with bromcresol green it appeared in many cases to be below 5. It is remarkable that enough acid apparently remained in the tooth to affect the indicators, even after the grinding process with distilled water washing over the sections. Since the demonstration depends on color change, it is difficult to show, save by color photographs. Owing to the expense involved, these were not possible, and it is hoped that the black and white photographs (Fig. 1), taken from the color transparencies, will at least give an indication of the staining zones. Further work is proceeding.

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COLLECTION OF MILLIGRAM QUANTITIES OF DENTAL ENAMEL DUST BY ELECTROSTATIC PRECIPITATION

LELAND C. HENDERSHOT*

Biochemical Research Laboratory, The Dow Chemical Company, Midland, Mich.

THE COLLECTION of enamel dust, ground from the surface of small teeth (for example, rat molars), presents some problems which are not encountered when similar operations are performed on larger teeth. Because the amounts of dust evolved are so small, the use of suction filters is impractical. However, the problem of collecting minute samples of enamel may be solved by the use of a small electrostatic precipitator of the type used by industrial hygienists of air sampling.

Fig. 1 shows the sampling head of such an instrument.† When the grinding operation is performed close to the mouth of the tube with the sampling head laid on the bench top, only negligible amounts of sample are lost. The ground dust is drawn into the sampling

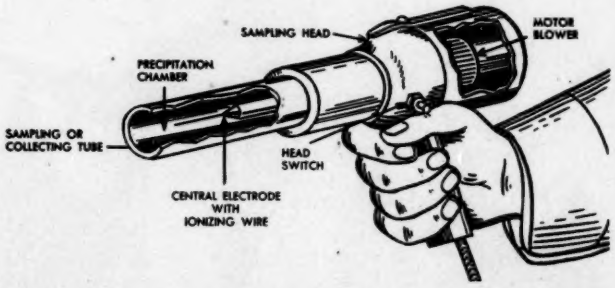

Fig. 1.—(Reprinted with the permission of the Mine Safety Appliances Co.)

tube by means of a small blower (capacity = 3 cubic feet/min.) and the dust particles are repelled to the wall of the tube by the high voltage central electrode. If the tube is lined with a piece of glassine paper‡ of suitable size (4⅜ by 5¾ in.), the removal of the sample is quite simple. The collected sample may be retained in the same paper used as the liner merely by scraping the dust together, then folding, sealing, and labeling the tiny packet. Repulsion from the high voltage electrode keeps the paper tightly pressed to the wall of the tube while the machine is in operation. Since virtually all the dust is precipitated before it has traveled 7 to 8 cm. through the tube, it is unnecessary to line the entire tube.

Samples of 1 to 4 mg. can be collected from a single rat by leaving the molars embedded in the bone and grinding the buccal and lingual enamel surfaces. Of course, pooled samples also may be collected. The handling of the specimens is aided by mounting each quadrant on a short piece of dowel stick with gem cutters' dop cement.

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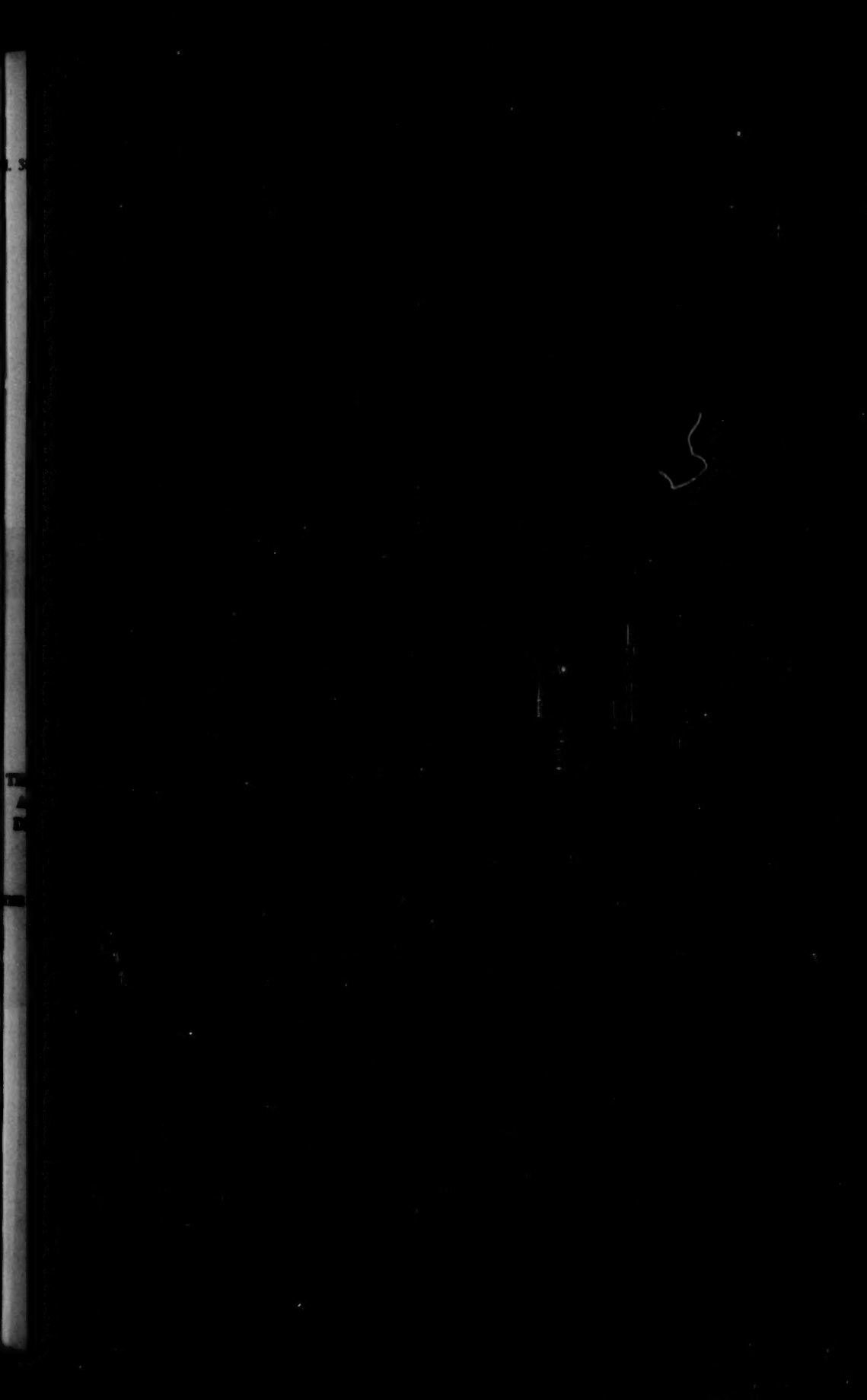
*Present address: American Dental Association, Chicago, Ill.

†Mine Safety Appliances Co., Pittsburgh, Pennsylvania.

‡Eli Lilly & Co., Indianapolis, Ind.